IMMUNOLOGICAL CORRELATION BETWEEN VARIOUS AUTOIMMUNE DISEASES AND *MYCOBACTERIUM TUBERCULOSIS*

ABSTRACT

THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

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BY

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ABSTRACT

Abstract

One of the hallmarks of immune system is the tremendous potentiality to discriminate between self and non-self. Systemic Lupus Erythematosus (SLE) - a prototype autoimmune disorder, is a chronic, life-long and potentially fatal disease, which is characterized by unpredictable exacerbations and remissions with protean clinical manifestations. SLE is a complex disorder affecting predominately young populations and shares similarities with HIV infection which is well known associated with tuberculosis (HIV-Tuberculosis) as regards the propensity for multiple organ involvement, potentially life-threatening episodes, and need for sophisticated monitoring. The clinical features of SLE are protean and may mimic infectious mononucleosis, lymphoma, or other systemic disease. The origin of autoantibody production in SLE is still poorly understood till date. However, a role has been suggested for an antigen driven process, spontaneous B-cell hyper-responsiveness, or impaired immune regulation. Thus, the etiology of SLE still remains poorly understood and warrants in-depth investigation for the management of the disease.

Evidences exist for the involvement of mycobacterial protein antigens in the pathogenesis of autoimmune disease. Furthermore, established evidences exist for autoimmune mediated destruction of islet cells of pancreas in the pathogenesis of Type-1 Diabetes mellitus for which various markers have been identified.

The direct and or indirect role of proinflammatory cytokine namely TNF-α in SLE has been indicated. TNF-α exerting its effects through its receptors namely TNFR-I and TNFR-II plays a crucial role in host defense. Inhibition of TNF-α clearly predisposes to certain infections, such as granulomatous infections like TB. Inhibition of TNF-α may also play a role in autoimmunity although the pathophysiologic mechanisms are uncertain. Furthermore, cases of TB have been reported in patients treated with TNF-α-antagonists. The risk for TB in RA/SLE patients is associated with multiple other factors including age, country of origin or current residence, exposure history to persons with TB, concomitant

therapy with other immunomodulators including corticosteroids, and disease activity. Focus has now shifted to development of compounds from natural sources that have anti-inflammatory and antimycobacterial activity. By boosting host immunologic responsiveness, these compounds may be particularly useful in the treatment of autoimmune disorders as well as drug-resistant tuberculosis. Our study involves the incorporation of such a compound, namely EGCG – a green tea polyphenol, as the natural herbal component for SLE and SLE-TB management.

Thus, the present study was carried out to probe the possible involvement of mycobacterial protein / nucleic acid antigen(s) acting as an alternate auto antigen for autoimmune disorders by employing various techniques such as direct binding and competition ELISA, cell culture with and without modulators, 'real time' RT-PCR, etc. Thus, it is hoped that the present study would help in the better understanding about the contributory role of *M. tuberculosis* infection in the morbidity and mortality of patients with SLE.

Cellular signalling by TNF-α is mediated mainly through activation of NF-κB. During characterization studies in order to assess the role of NF-κB in the expression of TNF-α in SLE, TB and SLE-TB monocytes, we employed SN50, an inhibitor of NF-κB. From our results, it was apparent that the induction of TNF-α expression was mediated through activation of NF-κB, because TNF- α was suppressed when SN50 was present in cultures. The inactive analogue of SN50 (i. e. SN50M) did not have any effect. Thus, as previously reported in macrophages, this study also shows that cellular activation is associated with augmentation of expression of both TNF-α in monocytes of patients with SLE, TB and SLE-TB.

Cell viability and potential cytotoxicity of EGCG, if any, on monocytes obtained from patients with SLE, TB and SLE-TB were determined for the concentrations (0-25 μg/ml) employed in this study using trypan blue and MTT assays, where viability of ~98-99% was observed. Interestingly, no effect of EGCG was observed on human housekeeping genes like R18 rRNA, thereby demonstrating

that the effect of EGCG was not mediated by cellular death, but rather by specific inhibition of expression as well as secretion of TNF-α.

To the best of our understanding, in our characterization studies, we show for the first time that EGCG exerts potent anti-inflammatory effects on host mononuclear cells obtained from patients of SLE, TB and SLE-TB, as evidenced by a strong inhibition of the pro-inflammatory cytokine TNF-α. The results indicate an appreciable suppression in soluble TNF-α secretion with EGCG.

TNF-α production in monocytes is regulated at multiple intracellular levels, beginning with transcription. Augmented expression of TNF-α mRNA and activation of a relevant transcription factor, NF-κB have been reported in monocytic cells infected with *M. tuberculosis*. Induction of TNF-α expression was mediated through activation of NF-κB, as evidenced by the suppression of secreted TNF-α protein in the presence of SN50, an inhibitor of NF-κB. On the contrary, SN50/M, an inactive analogue of SN50, failed to show any such effect. It has been well established that TNF-α induced nuclear translocation of NF-κB is inhibited by SN50 peptide as demonstrated in EMSA. In view of it, our data demonstrated that this effect involved inhibition of the NF-κB pathway induced by EGCG, probably by inhibiting the degradation of IκBα. The NF-κB heterodimer is retained in the cytoplasm in an inactive form through association with one of the IκBs inhibitory proteins.

In continuation to the above, a role for the antioxidant enzyme glutathione peroxidase (GPx) in MTB-infected and autoimmune mononuclear cells was investigated with respect to various modulators employed in this study. In the present study, reduced / suppressed GPx activity in patient monocytes that were untreated or treated with SN50/M was recorded, thereby concurring with earlier reports that substantial amounts of ROS are being generated in patient cells due to cellular activation. Enhancement of GPx activity in monocyte cultures from patients with SLE, TB and SLE-TB after addition of NAC, a precursor of the *in vivo* antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, GPx activity was observed to be further augmented

when EGCG was co-cultured instead of NAC, indicating EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in mononuclear phagocytes. Moreover, in continuation to the above, our data further shows the lipid peroxidation-induced augmented MDA levels in culture supernatants of monocytes from patients with SLE, TB and SLE-TB were appreciably suppressed or down-regulated with EGCG.

Our results are strongly indicative for the appreciable correlation between autoimmune diseases like SLE and *Mycobacterium tuberculosis* infection. Inhibition ELISA results strongly suggests that all the TB positive sera selected in this study were having a high degree of specificity towards MTB Ag85B (30kDa). Similarly, our data show high degree of recognition of anti-DNA antibodies found in SLE sera by native dsDNA. The achievement of 50% inhibition in antibody activity in both the above diseases i. e. TB and SLE at a very low inhibitor concentrations are indicative for the presence of highly specific respective antibodies in all the sera selected for this study.

One of the interesting findings of this study was that the SLE patients were found to be more susceptible to TB development than the vice-versa case. This is evident from the data indicating that autoantibodies found in SLE sera strongly recognized both native dsDNA as well as mycobacterial Ag85B 30kDa, whereas anti-TB antibodies found in TB sera strongly recognized only Ag85B 30kDa, whereas it exhibited low or negligible recognition with native dsDNA. However, antibodies found in sera of patients suffering with both TB along with SLE (SLE-TB) revealed high specificity for both the antigens i. e. native dsDNA and MTB 30 kDa.

An attempt was also made to evaluate the immuno-binding by generating in-vivo conditions i. e. by using cultured monocytes that were infected with bacilli (TB patients) as well as monocytes from SLE patients. The high percent maximum inhibitions as well 50% inhibitions in anti-TB and anti-DNA activities at extremely low inhibitor concentrations are suggestive for the appreciably high affinity

immuno-interaction occurring between anti-TB antibodies and anti-DNA autoantibodies with antigens in monocyte protein lysates as well as respective dsDNA of TB patients and SLE patients respectively.

A striking finding in the present study is the EGCG as well as reduced glutathione-induced down-regulation in binding of anti-TB antibodies and anti-DNA antibodies with antigens present in protein lysates prepared from monocyte of TB patients and SLE patients respectively. Similar actions of reduced glutathione in monocytes obtained from blood of patients having both TB along with SLE further substantiates the antioxidant-induced suppression in immuno-binding. Furthermore, these results are correlating appreciably with the amount of TNF-α secreted in different monocyte cultures undertaken in this study.

To have further in-sight, the present study involves utilization of dsDNA isolated from monocyte cultures of SLE, TB and SLE-TB patients that were either untreated or treated with EGCG, NAC, SN50 and SN50M, an in turn, employed as antigens / inhibitors in immunoassays against anti-DNA SLE antibodies and anti-TB antibodies respectively. Thus, interesting important observations were made. We observed that purified anti-DNA antibodies from SLE patients exhibited high degree of recognition / specificity against dsDNA isolated from monocytes of patients with SLE, TB and SLE-TB respectively. However, this high magnitude specificity / binding of purified anti-DNA antibodies from SLE patients was reduced or suppressed appreciably towards respective dsDNA isolated from monocytes of patients with SLE, TB and SLE-TB respectively that were treated with EGCG, NAC *or SN50*. On the contrary, anti-TB antibodies exhibited high binding only with dsDNA isolated from monocytes of patients with TB, but failed to show any significant recognition / binding with dsDNA isolated from monocytes of patients with SLE. Furthermore, when monocyte cultures of SLE, TB and SLE-TB patients that were treated with EGCG, NAC and SN50, and in turn, respective dsDNA isolated and employed in ELISA, reduced or insignificant binding was observed against anti-dsDNA antibodies from SLE patients or anti-TB antibodies from TB patients. Thus, in view of the fact that native DNA is a

poor immunogen, and that, the exact trigger of anti-DNA production in SLE still remains poorly understood, where DNA has been thought to act only as a cross-reacting antigen, the results indicate possible involvement of Mycobacterium tuberculosis protein(s) / nucleic acid antigens(s) in providing an alternate trigger / origin for autoantibody production in systemic lupus erythematosus (SLE). Moreover, the data generated in the present study is suggestive for the fact that – reactive oxygen species (ROS) generated as a consequence of stress of any kind in autoimmune SLE results in the activation of proinflammatory cytokine TNF-α, which in turn results in the production of anti-DNA auto antibodies. These SLE patients having high ROS levels become highly susceptible to MTB infection. Upon MTB infection, the ROS and TNF-α potentiates or activates the MTB 85B replication in SLE-TB patients. Such an activation of ROS or TNF-α and in turn the SLE and TB proliferation could be arrested or limited by the usage of EGCG- a green tea polyphenol and reduced glutathione as revealed by our data. In conclusion, based on characterization studies, followed by immunological data, it can be inferred from the present study that:

1. There exist high basal levels of TNF-α in sera as well as in monocyte cultures of patients with SLE, TB and SLE-TB.
2. Activation of monocytes by *M. tuberculosis* infection in SLE patients induces the expression of both TNF-α at both the gene and protein levels.
3. Both RNI and ROI, induced early after infection of SLE monocytes, increases expression of TNF-α.
4. Activation of monocytes by *M. tuberculosis* initiates a cascade of events whereby a vicious circle may exist in which expression of host inflammation and mycobacterial products amplify one another.
5. EGCG (0-25 μg/ml) exhibited no toxic effect on the viability of human monocytes.
6. EGCG inhibits the expression of TNF-α protein production in a dose-dependent manner in 24 hr monocyte cultures from patients with SLE, TB and SLE-TB, and that, it is mediated mainly via NF-κB.

7. EGCG ameliorates the glutathione peroxidase activity in monocytes from patients with SLE, TB and SLE-TB.
8. EGCG suppresses the augmented MDA levels in monocytes from patients with SLE, TB and SLE-TB.
9. All the *M. tuberculosis* and SLE sera involved in this study showed a high degree of specificity for Ag85B (30 kDa) and native dsDNA respectively.
10. Mycobacterial 30 kDa protein antigen (Ag85B) as well as protein lysates prepared from monocytes of *M. tuberculosis* patients were recognized appreciably by anti-tuberculosis antibodies present in *M. tuberculosis* sera, whereas non-mycobacterial native dsDNA showed poor recognition with the same anti-tuberculosis antibodies.
11. On the contrary, both non-mycobacterial native dsDNA and protein lysates prepared from monocytes of SLE patients as well as mycobacterial 30 kDa protein antigen (Ag85B) were found to be recognized appreciably by anti-DNA autoantibodies present in SLE sera.
12. Furthermore, co-culturing of monocytes obtained from *M. tuberculosis*, SLE or *M. tuberculosis*-SLE with 10 nM of reduced glutathione showed amelioration of ROS and TNF-α induced actions, which in turn, subsequently suppressed the immuno-bindings observed in monocytes of *M. tuberculosis* and SLE patients cultured without glutathione.
13. Our data shows that SLE patients are more susceptible to *M.tuberculosis* development, as ROS and TNF-α in SLE patients could activate the replication of Ag85B (30 kDa) after bacilli infection.
14. Finally, immunoaffinity purified anti-DNA antibodies from SLE patients recognized dsDNA isolated from monocytes of both SLE and TB patients, but on the contrary, anti-TB antibodies recognized dsDNA only from monocytes from TB patients but not SLE patients.

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ALIGARH (INDIA)

2008

Dedicated

to

my

Parents

DEPARTMENT OF BIOCHEMISTRY
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Date: 20th April, 2008

Certificate

*This is to certify that the research work and techniques incorporated in this thesis for Ph. D entitled "**Immunological Correlation Between Various Autoimmune** [redacted] **tuberculosis**" are the bonafide work of Miss Nazia [redacted] herself under my strict guidance and direct supervision has undertaken the study. The techniques and observations embodied in this thesis have been checked at every stage.*

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LIST OF ABBREVIATIONS

BCG	:	Bacille Calmette-Guerin
BSA	:	Bovine serum albumin
cDNA	:	Complementary **DNA**
DEPC	:	Diethyl pyrocarbonate
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate,
DOTS	:	Direct'y Observed Treatment Strategy
DTT	:	Dithiothreiotol
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked imminosorbent assay
EMSA	:	Electrophoretic mobiliy shift assay
GPx	:	Glutathione peroxidase
GSSG	:	Oxidized glutathione
H_2O_2	:	Hydrogen peroxide
IC50	:	Concentration for 50% inhibition
IFN-γ	:	Interferon-gamma
IgG	:	Immunoglobulin G
IL	:	Interleukin
LAM	:	Lipoarabinomannan
LPS	:	Lipopolysaccharide
MDR	:	Multidrug-resistant
MHC	:	Major histocompatibility complex
ml	:	Milliliter
mM	:	Millimolar
MN	:	Monocyte
mRNA	:	Massanger RNA
MTCF	:	*Mycobacterium tuberculosis* culture filtrate
MTSE	:	*Mycobacterium tuberculosis* sonic extract
MTT	:	3-(4, 5-dimethylthiazol-2-yl)-2, diphenyltetrazolium bromide
NAC	:	N-acetyl cysteine
NADPH	:	Reduced β-nicotinamide adenine dinucleotide phosphate

NF-xB	:	Nuclear factor kappa B
NHS	:	Normal human serum
nM	:	Nanomolar
NMMA	:	N^G-monomethyl-L-arginine-monoacetate
NO	:	Nitric oxide
NOC-9	:	Nonoate-9
oATP	:	Oxidized ATP
PAGE	:	Polyacrylamide gel electrophoresis
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffer saline
PMSF	:	Phenylmethylsulphonyl fluoride
pNPP	:	p-nitrophenyl phosphate
rh	:	Recombinant human
RNA	:	Ribonucleic acid
RNI	:	Reactive nitrogen intermediate
ROI	:	Reactive oxygen intermediate
ROS	:	Reactive oxygen species
RPMI	:	Roswell Park Memorial Institute
rRNA	:	Ribosomal RNA
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SDS	:	Sodium dodecyl sulphate
SN50	:	An inhibitor of NF-κB
SNP	:	Sodium nitroprusside
sTNFR	:	Soluble TNF receptor
TDM	:	Trehalose dimycolate
TEMED	:	N,N,N',N'-tetramethylethylene diamine
TLR	:	Toll-like receptor
TMM	:	Trehalose monomycolates
TNF-α	:	Tumor necrosis factor-alpha
Tris	:	Tris (hydroxymethyl) aminomethane
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar

Acknowledgements

First and foremost I thank "ALMIGHTY GOD" the most merciful and beneficent, for bringing me this far in life.

With sincere feelings I express my deep sense of gratitude, benevolence and thankfulness to my esteemed supervisor ***DR. NAJMUL ISLAM*** *.It has been his constant support encouragement, enthusiasm and scholarly guidance that led to the completion of this work.*

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It won't be a eulogy if I call my parents an incarceration of God on this earth .They made me dream - and nourished it to reality.

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Lastly, but never the least, I thank my Alma Mator, Aligarh Muslim University, Aligarh, the great seat of learning, in nurturing me as well as providing all necessary help in imbibing knowledge during the course of my education.

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CONTENTS

ABSTRACT

Abstract

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1. There exist high basal levels of TNF-α in sera as well as in monocyte cultures of patients with SLE, TB and SLE-TB.
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3. Both RNI and ROI, induced early after infection of SLE monocytes, increases expression of TNF-α.
4. Activation of monocytes by *M. tuberculosis* initiates a cascade of events whereby a vicious circle may exist in which expression of host inflammation and mycobacterial products amplify one another.
5. EGCG (0-25 μg/ml) exhibited no toxic effect on the viability of human monocytes.
6. EGCG inhibits the expression of TNF-α protein production in a dose-dependent manner in 24 hr monocyte cultures from patients with SLE, TB and SLE-TB, and that, it is mediated mainly via NF-κB.

7. EGCG ameliorates the glutathione peroxidase activity in monocytes from patients with SLE, TB and SLE-TB.
8. EGCG suppresses the augmented MDA levels in monocytes from patients with SLE, TB and SLE-TB.
9. All the *M. tuberculosis* and SLE sera involved in this study showed a high degree of specificity for Ag85B (30 kDa) and native dsDNA respectively.
10. Mycobacterial 30 kDa protein antigen (Ag85B) as well as protein lysates prepared from monocytes of *M. tuberculosis* patients were recognized appreciably by anti-tuberculosis antibodies present in *M. tuberculosis* sera, whereas non-mycobacterial native dsDNA showed poor recognition with the same anti-tuberculosis antibodies.
11. On the contrary, both non-mycobacterial native dsDNA and protein lysates prepared from monocytes of SLE patients as well as mycobacterial 30 kDa protein antigen (Ag85B) were found to be recognized appreciably by anti-DNA autoantibodies present in SLE sera.
12. Furthermore, co-culturing of monocytes obtained from *M. tuberculosis*, SLE or *M. tuberculosis*-SLE with 10 nM of reduced glutathione showed amelioration of ROS and TNF-α induced actions, which in turn, subsequently suppressed the immuno-bindings observed in monocytes of *M. tuberculosis* and SLE patients cultured without glutathione.
13. Our data shows that SLE patients are more susceptible to *M.tuberculosis* development, as ROS and TNF-α in SLE patients could activate the replication of Ag85B (30 kDa) after bacilli infection.
14. Finally, immunoaffinity purified anti-DNA antibodies from SLE patients recognized dsDNA isolated from monocytes of both SLE and TB patients, but on the contrary, anti-TB antibodies recognized dsDNA only from monocytes from TB patients but not SLE patients.

LIST OF ILLUSTRATIONS

INTRODUCTION

INTRODUCTION

The central dogma of immunology for many years was focused on the clonal detection of auto reactive cells, thereby, leaving a repertoire of T cells that recognize specific foreign antigen. However, with the advancement in the field of immunology, it was acknowledged that a low level of autoreactivity is physiologic (Dighiero et al., 1999) and crucial to normal immune function. Autoantigen helps to form the repertoire of mature lymphocytes, and the survival of naïve T cells (Goldrath et al., 1999) and B cells (Gu et al., 1991) in the periphery requires continuous exposure to auto antigens. Since there is no fundamental difference between the structure of self antigens (auto antigens) and that of foreign antigens, lymphocytes evolved not to distinguish self from foreign, as some have speculated, but to respond to antigen only in certain microenvironments, generally in the presence of inflammatory cytokines (Silverstein et al., 2000). Since autoreactivity is physiologic, the challenge is to understand how it becomes a pathologic process and how T cells and B cells contribute to tissue injury.

One of the hallmarks of immune system is the tremendous potentiality to discriminate between self and non-self. However, this enormous recognition potential includes possible interaction with self components, some of which may appear to be essential for the regulation of the immune functions whereas others can be pathogenic, particularly if expressed at high level, leading to autoimmune diseases.

Systemic Lupus Erythematosus (SLE) - a prototype autoimmune disorder, is a chronic, life-long and potentially fatal disease, which is characterized by unpredictable exacerbations and remissions with protean clinical manifestations. In SLE, there is a predilection for clinical involvement of the joints, skin, kidney, brain, serosa, lung, heart and gastrointestinal tract. Moreover, SLE is a complex disorder affecting predominately young populations and shares similarities with HIV infection which is well known associated with tuberculosis (HIV-Tuberculosis) as regards the propensity for multiple organ involvement,

potentially life-threatening episodes, and need for sophisticated monitoring. The clinical features of SLE are protean and may mimic infectious mononucleosis, lymphoma, or other systemic disease. The origin of autoantibody production in SLE is still poorly understood till date. However, a role has been suggested for an antigen driven process, spontaneous B-cell hyper-responsiveness, or impaired immune regulation. Thus, the etiology of SLE still remains poorly understood and warrants in-depth investigation for the management of the disease.

Evidences exist for the involvement of mycobacterial protein antigens in the pathogenesis of autoimmune disease (Tasneem et al., 2001). Furthermore, established evidences exist for autoimmune mediated destruction of islet cells of pancreas in the pathogenesis of Type-1 Diabetes mellitus for which various markers have been identified.

The direct and or indirect role of proinflammatory cytokine namely TNF-α in SLE has been indicated. TNF-α exerting its effects through its receptors namely TNFR-I and TNFR-II is a central mediator of inflammation, immunity and autoimmunity and that, it plays a crucial role in host defense. Inhibition of TNF-α clearly predisposes to certain infections, such as granulomatous infections like TB (Islam et al., 2004; Wilkinson et al., 2001). Inhibition of TNF-α may also play a role in autoimmunity although the pathophysiologic mechanisms are uncertain. Furthermore, cases of TB have been reported in patients treated with TNF-α-antagonists. The risk for TB in RA/SLE patients is associated with multiple other factors including age, country of origin or current residence, exposure history to persons with TB, concomitant therapy with other immunomodulators including corticosteroids, and disease activity. TNF-α antagonists have been associated with an increase in the percentage of RA patients with positive serologies (ANA and anti-ds-DNA) and lupus-like syndromes. It is to be pointed out that the role of TNF-α in patients with osteoarthritis, which is an important form of arthritis as well as novel therapeutic strategies for the management of osteoarthritis has been well documented (Islam et al., 2002; Malemud et al., 2003).

In view of the above, the present study was carried out to probe the possible involvement of mycobacterial protein / nucleic acid antigen(s) acting as an alternate auto antigen for autoimmune disorders by employing various techniques such as SDS-PAGE, direct binding and competition ELISA, cell culture with and without modulators, 'real time' RT-PCR, etc. Thus, it is hoped that the present study would help in the better understanding about the contributory role of *M. tuberculosis* infection in the morbidity and mortality of patients with SLE.

AUTOIMMUNE DISORDERS AND ITS BASIS

For clinicians, autoimmune diseases appear to be either systemic (as in the case of systemic lupus erythematosus) or organ-specific (as in the case of type 1 diabetes mellitus). This classification, although clinically useful, does not necessarily correspond to a difference in causation. Alterations that lower the threshold for the survival and activation of autoreactive B cells, often cause the production of multiple autoantibodies, as in the case of the antinuclear and anti-DNA antibodies in systemic lupus erythematosus (Gorelik et al., 2000; Majeti et al., 2000). Genetic alterations with global effects on the function of regulatory T cells or cytokine production often lead to inflammatory bowel disease. (Bhan et al., 1999; Blumberg et al., 1999; Boismenu and Chen, 2000).

Environmental Triggers

Even in a genetically predisposed person, some trigger – an environmental exposure or a change in the internal environment – is usually required for frank auto reactivity. Studies of genetically similar populations living in different conditions strongly suggest the importance of environmental triggers. For example, the incidence of both type 1 diabetes and multiple sclerosis in a population changes as the members migrate to different regions (Noseworthy et al., 2000; Dahlquist, 1998).

Infectious Agents

Microbial antigens have the potential to initiate autoreactivity through molecular mimicry, polyclonal activation, or the release of previously sequestered antigens, for example, in autoimmune diabetes, T cells recognize both a peptide derived from the autoantigen glutamic acid dcecarboxylase and a highly analogous peptide from coxsackievirus P2-C protein (Kukreja et al., 2000).

Microbial infection can also cause polyclonal activation of autoreactive lymphocytes. This is presumed to be the mechanism underlying the increased incidence of autoimmune disease in rodents exposed to microbial pathogens (Horwitz et al., 1998). Microbes that kill cells can cause inflammation, release of sequestered antigens, resulting in autoimmunity (Horwitz et al., 1998; Miller et al., 1997).

Noninfectious Triggers

Many autoimmune diseases are much common in women than in men, and estrogens exacerbate systemic lupus erythematosus in murine models of the disease by altering the B-cell repertoire in the absence of inflammation (Bynoe et al., 2000). There is mounting evidence that blockade of TNF-α, which is beneficial in Crohn's disease and rheumatoid arthritis, can induce antinuclear antibodies and perhaps even systemic lupus erythematosus and multiple sclerosis in certain persons (Charles et al., 2000; Mohan et al., 2000). TNF-α has inhibitory effects on activated T cells, (Cope et al., 1997; Kassiotis and Kollias, 2001), but how it induces autoimmunity is unknown.

Loss of Regulatory Cells

Several kinds of regulatory cells are important in controlling autoreactivity: CD1-restricted T cells, T cells with / receptors, CD4+CD25+ T cells, and T cells that produce cytokines that suppress pathogenic autoreactive cells. Some of these regulatory cells – for example, CD4+CD25+ T cells – must mature in the thymus (Shevach, 2000); others require activation by autoantigens in the periphery.

Alterations in the number and function of regulatory cells may contribute to autoimmunization. In monozygotic twins who are discordant for diabetes, for instance, levels of CD1-restricted T cells are greatly diminished in the affected twin (Wilson et al., 1998).

DISEASE PROGRESSION

Epitope Spreading

As an autoimmune disease progresses from initial activation to a chronic state there is often an increase in the number of autoantigens targeted by T cells and antibodies ("epitope spreading") (Moudgil and Sercarz, 1994; Lanzavecchia, 1995), and, in some cases, a change in participating cells, cytokines, and other inflammatory mediators. Both autoreactive T cells and B cells contribute to epitope spreading.

Pathogenic Mechanisms

It has become apparent, primarily through studies in animals, that different effector cells and inflammatory mediators as the disease progresses may supersede the initial mechanisms causing autoreactivity in an autoimmune disease. Naïve lymphocytes are activated at the initiation of disease and may continue to be recruited by epitope spreading later in the disease, but it is unknown whether naïve cells or memory cells cause progression and flares of disease.

The fact that the cells and soluble mediators of injury can change over time has tremendous implications for therapy; interventions that are effective early may be less efficacious later on or may even be harmful. The unpredictability of these effects is amply illustrated by the clinical efficacy of the blockade of TNF-α in rheumatoid arthritis and Crohn's disease, at the cost of including antinuclear antibodies in up to 10 percent of treated patients and systemic lupus erythbematosus in a few patients.

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is defined by its clinical features and by the almost invariable presence of antibodies in blood / sera, directed against one or more components of cell nuclei. Certain manifestations seem to be associated with the presence of different antinuclear antibodies and genetic markers, which suggests that SLE may be a family of diseases. However, clinical presentations frequently overlap, and there is currently no conclusive evidence that SLE is more than a single disorder with a broad range of manifestations, the expression of which may be influenced by the patient's genetic background. Occasionally patients have features of both SLE and other connective-tissue diseases, such as rheumatoid arthritis and scleroderma.

Antinuclear antibodies have not been shown to be directly involved in the pathogenesis of the disease, but patients with SLE often have other circulating antibodies that react with cell membranes or serum components. Some are directly responsible for clinical manifestations; others may play a part in the immune dysregulation that underlies the disease. The mechanisms that result in such prolific antibody production are a focus of intense research.

The prevalence of SLE varies throughout the world. In North America and northern Europe, it is about 40 per 100,000 population (Hochberg, 1990). Over 80 percent of cases occur in women during their childbearing years. As a consequence, the disease may affect as many as 1 in 1000 young women. The prevalence in children and older adults is approximately 1 per 100,000, with a ratio of female to male patients of 3:1.

Diagnosis

Polyarthritis and dermatitis are the most common clinical manifestations of SLE, and the most common presenting symptoms (Pistiner et al., 1991). However, any symptom or sign of the disease may be its first manifestation, and a single one, such as arthritis, thrombocytopenia, or pericarditis, may persist or recur for months or years before the diagnosis can be confirmed by the appearance of

other features. Chronic fatigue and a variety of disturbances of cognition or affect including anxiety and depression are frequently described by patients as early symptoms (Iverson, 1993). Isolated major involvement of the kidney or central nervous system is unusual. Criteria to distinguish SLE from other connective-tissue diseases have been established by the American Rheumatism Association and were revised in 1982 and 1997 (Tan et al., 1982; Hochberg, 1997).

The detection of antinuclear antibodies is a sensitive screening test for SLE. Since antinuclear antibodies occur in more than 95 percent of patients, (Hochberg, 1990) it is hard to be certain of the diagnosis in their absence. The most common antibody in patients with the disease is directed against nucleosomal DNA-histone complexes, and it yields a homogeneous staining pattern on the immunofluorescence test for antinuclear antibodies (Mohan et al., 1993). Antinuclear antibody also occurs in most of the other rheumatic diseases, autoimmune liver and thyroid diseases, and some drug reactions (Tan, 1989). It is produced transiently in viral infections and is present, usually in low titers, in about 2 percent of the normal population. The degree of positivity of the anti-nuclear-antibody test is diagnostically important. Serum dilutions below which normal serum may be positive for these antibodies vary in different laboratories. Titers that are less than two times higher than the normal limit in any laboratory should be viewed skeptically. The positive predictive value of the test increases with higher titers.

Antibodies to native or double-stranded DNA and to Sm, a ribonuclear protein antigen, are more specific than other antinuclear antibodies for the diagnosis of SLE. Their presence does not predict particular disease manifestations, although nephritis is more common in patients with antinative DNA (Swaak et al., 1986). For many of the patients who have anti-native DNA, the titer of this antibody is a useful measure of disease activity (Borg et al., 1990).

Clinical Course of SLE

The natural history of SLE is highly variable and unpredictable. The 10-year survival rate for patients observed over the past decade approaches 90 percent

(Pistiner et al., 1991). The presence of nephritis and systolic hypertension indicates a poorer prognosis (Seleznick and Fries, 1991). The risk of life-threatening complications, particularly nephritis, appears to be greater during the first five years after the onset of the disease and is associated with the presence of antibody to native DNA and younger age (Swaak et al., 1989). The prognosis for men and children with SLE is less favorable than it is for women (Miller et al., 1983; Barron et al., 1993). SLE that begins in either sex after the age of 60 tends to have a more benign course; arthritis, pleurisy, rash, and anemia are usually the major manifestations (Baker et al., 1979).

Antiphospholipid antibody can be detected in approximately 30 percent of patients with SLE (Love and Santoro, 1990). It is responsible for a wide variety of thromboembolic complications, such as stroke, portal-vein thrombosis, thrombophlebitis, and pulmonary embolism (McHugh et al., 1991). For these reasons, it is an important determinant of disease outcome (Lockshin, 1993). The risk of thromboembolic complications is not the same for all patients who have antiphospholipid antibody, and no single test to detect the antibodies can reliably predict the risk. The risk seems to be greater in patients who have positive results on one of the tests assessing function, such as the diluted-thromboplastin time. A recently identified cofactor may be important (Ordi et al., 1993). Acute infections can lead to a positive result on immunoassay that is not dependent on the cofactor.

NATURAL HISTORY OF TUBERCULOSIS

Agent factors

M. tuberculosis is a facultative intracellular parasite, it is readily ingested by phagocytes and is resistant to intracellular killing. Of importance to man are the human and bovine strains. The human virulent strain ($H_{37}R_v$) is responsible for the vast majority of cases. The bovine strain affects mainly cattle and other animals. Regarding virulence, the Indian tubercle bacillus is said to be less

virulent than the European bacillus. In recent year, a number of mycobacteria have been isolated from human which have been classified into 4 groups – (i) photocromogens (e.g. M. Kansasii); (ii) scotochromogens (e.g. M. scrofulaceum); (iii) non-photocromogens (e.g. M. intercellular) and (iv) rapid growers (e.g. M. fortuitum). All these are mainly saprophytic. Diseases attributed to them have resembled pulmonary tuberculosis and chronic cervical lymphadenitis. Non-specific infections have been reported to be widely prevalent in the southern part of India (Park, 2005).

Source of Infection: There are two sources of infection; human and bovine.

(i) Human source: The most common source of infection is the human case whose sputum is positive for tubercle bacilli and who has either received no treatment or partially treated.

(ii) Bovine source: The bovine source of infection is usually infected milk.

Communicability: Patients are infected as long as they remain untreated.

Host factors:

Age: Tuberculosis affects all ages. In the developed countries, the disease is more common in the elderly.

Sex: More prevalent in males than females.

Heredity: Tuberculosis is not a heredity disease. However twin studies indicate that inherited susceptibility is an important risk factor.

Nutrition: Malnutrition is widely believed to predisposed to tuberculosis, but the available evidence on this point is only indirect.

Immunity: Man has no inherited immunity against tuberculosis. It is acquired as result of natural infection or BCG vaccination.

Social Factors

Tuberculosis is a social disease with medical aspects. It has also being described as a barometer of social welfare. The social factors include many non medical factors such as poor quality of life, poor housing and over crowding, population explosion, lack of education etc. (Park, 2005).

Mode of Transmission

Tuberculosis is transmitted mainly by droplet infection and droplet nuclei generated by sputum positive patients with pulmonary tuberculosis.

Incubation Period

The time from receipt of infection to the development of a positive tuberculin test ranges from 3 – 6 weeks and thereafter, the development of disease depends upon the closeness of contact, extent of the disease and sputum positivity of the source case and host parasite relationship (Park, 2005).

Cytokine production driven by *M. tuberculosis*:

Cytokines are a group of hormone-like polypeptides that play a variety of regulatory roles in host defense against infection. Once infection is established, a focal nonspecific inflammatory response follows. This response is regulated by a network of pro- and anti-inflammatory cytokines and chemokines (Crevel et al., 2002), which serve as signals of infection (Means et al., 1999). There are two kinds of cytokines, pro-inflammatory and anti-inflammatory. The pro-inflammatory cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial function. This response is antagonized by anti-inflammatory mechanisms. In addition some cytokines may inhibit the production or the effects of pro-inflammatory cytokines in tuberculosis (Crevel et al., 2002).

(a) Pro-inflammatory cytokines:

(i) TNF-α:

Stimulation of monocytes, macrophages and dendritic cells with mycobacteria or mycobacterial products induces TNF-α production (Henderson et al., 1997;

Valone et al., 1988). This prototype proinflammatory cytokine plays a key role in granuloma formation (Senaldi et al., 1996), induces macrophage activation and has immunoregulatory properties (Tsenova et al., 1999). However, systemic spillover of TNF-α may account for unwanted inflammatory effects like fever and wasting (Crevel et al., 2002). Thus, TNF-α has both adverse and beneficial effects in the human immune response (Tufariello et al., 2003). To limit the deleterious effects of TNF-α (Hernandez and Rook, 1994; Bekker et al., 2000), systemic production of TNF-α is downregulated (Friedland et al., 1995; Takashima et al., 1990). In spite of this, anti-TNF-α therapy in the treatment of tuberculosis is associated with an increased risk of developing tuberculosis (Keane and Gershon, 2002), mainly due to reactivation of a chronic infection (Keane et al., 2001). In addition, TNF-α has an important role in the maintenance of the granulomatous response in latent tuberculosis, and in maintaining the organization of the granuloma, the integrity of which contributes to the containment of *M. tuberculosis* (Tufariello et al., 2003).

(ii) IL-1β:

IL-1β, a pro-inflammatory cytokine is produced mainly by monocytes, macrophages and dendritic cells (Dahl et al., 1996; Gerosa et al., 1999). It is expressed in excess (Schauf et al., 1993) and at the site of disease in tuberculosis patients (Bergeron et al., 1997). IL-1R antagonist (IL-1Ra) is the naturally occurring antagonist of IL-1. It has been suggested that an increased IL-1β/IL-1Ra ratio protects against a more severe presentation of tuberculosis, especially tuberculosis pleuritis (Wilkinson et al., 1999).

(iii) IL-6:

This is an early cytokine, which has both pro- and anti-inflammatory properties (VanHeyningen et. al., 1997) and is produced at the site of infection (Hoheisel et al., 1998). IL-6 may be harmful in mycobacterial infections, as it inhibits the production of TNF-α and IL-1β (Schindler et al., 1990). However, other reports indicate a protective role for IL-6, which seems related to optimal IFN-γ production early in *M. tuberculosis* infection (Ladel et al., 1997a).

(iv) IL-12:

Mainly phagocytic cells produce IL-12 after phagocytosis of *M. tuberculosis* (Ladel et al., 1997b). It is an important early pro-inflammatory cytokine that drives the production of IFN-γ from natural killer (NK) cells (Trinchieri, 2003). Thus, IL-12 is the inducer cytokine and IFN-γ is the effector cytokine that mediates protection (Ismail et al., 2002) as discussed further. IL-12 is a regulatory cytokine that connects the innate and adaptive host response to mycobacteria (Trinchieri, 1995) and which exerts its protective effects mainly through the induction of IFN-γ (Cooper et al., 1997). In tuberculosis, IL-12 has been detected in lung infiltrates (Taha et al., 1997), and in granulomas (Bergeron et al., 1997). The expression of IL-12 receptors is also increased at the site of disease (Zhang et al., 1999). Three additional cytokines with IL-12-like activities have been identified, notably IL-18, IL-23 and IL-27 (Vosse et al., 2004). IL-18, a novel pro-inflammatory cytokine shares many features with IL-1 (Dinarello et al., 1998) and acts as an IFN-γ inducing factor, synergistic with IL-12 (O'Neill and Greene, 1998). It also stimulates the production of other pro-inflammatory cytokines, chemokines, and transcription factors (Netea et al., 2000). IL-15 is similar to IL-2 in its biological activities and stimulates T cell and NK cell proliferation and activation (Kennedy and Park, 1996). However, unlike IL-2, the production of IL-15 is mainly by monocytes and macrophages (Crevel et al., 2002).

(v) IFN-γ:

An important macrophage-activating molecule involved in the immune defense against pathogenic mycobacteria is interferon-γ. Production of IFN-γ is regulated by other cytokines, particularly IL-12. In the early phase of the immune response, IL-12 drives the production of IFN-γ from NK cells (Trinchieri, 2003). Several immune mechanisms, such as antigen presentation, leukocyte-endothelium cell interactions, cell growth and apoptosis, reactive nitrogen and oxygen intermediates as well as phagosome-lysosome fusion can be modulated through the activity of IFN-γ (Houben et al., 2006). Activation of macrophages by IFN-γ

also induces autophagy, which is a normal degradative pathway implicated in innate immune mechanisms against *M. tuberculosis* (Ogawa et al., 2005).

(b) Anti-inflammatory cytokines:

The pro-inflammatory response is antagonized by anti-inflammatory mechanisms. Soluble cytokine receptors prevent binding of cytokines to cellular receptors, blocking further signalling (Crevel et al., 2002). Anti-inflammatory cytokines are also important in immuno-regulation.

(i) IL-10:

IL-10 is produced by macrophages after *M. tuberculosis* phagocytosis (Shaw et al., 2000) and after binding of LAM (Dahl et al., 1996), by dendritic cells and macrophages (Tufariello et al., 2003). IL-10 downregulates the production of pro-inflammatory cytokines IFN-γ, TNF-α and IL-12 (Fulton et al., 1998; Hirsch et al., 1999). IL-10 can deactivate macrophages and dampen the immune response to prevent or limit pathology from an over-exuberant inflammatory response to a pathogen. This cytokine may have a role in the chronic phase of infection, since downregulation of a type I immune response is likely to be beneficial to the host, at least in terms of lung pathology (Tufariello et al., 2003). IL-10 expression in tuberculosis has been demonstrated in circulating mononuclear cells, at the site of disease in pleural fluid and in alveolar lavage fluid (Barnes et al., 1993; Gerosa et al., 1999).

(ii) TGF-β:

TGF-β is produced by monocytes and dendritic cells following induction by mycobacterial products (Toossi et al., 1995). TGF-β suppresses cell-mediated immunity; in T cells it inhibits proliferation and IFN-γ production; in macrophages it antagonizes antigen presentation, pro-inflammatory cytokine production, and cellular activation. TGF-β may be involved in tissue damage and fibrosis during tuberculosis, as it promotes the production and deposition of macrophage collagenases (Toossi and Ellner, 1998).

(iii) IL-4:

IL-4 has deleterious effects in tuberculosis, which have been ascribed to this cytokine's suppression of IFN-γ production (Powrie and Coffman, 1993) and macrophage activation (Appelberg et al., 1992).

NF-κB activation – Role in innate immunity:

It is well established that the classical NF-κB pathway, based on IKKβ-dependent IκB degradation, is essential for innate immunity (Bonizzi and Karin, 2004). The activation and nuclear translocation of classical NF-κB dimers is associated with increased transcription of genes encoding chemokines, cytokines, adhesion molecules [intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leukocyte adhesion molecule-1 (ELAM-1)], enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis (Ghosh et al., 1998). These molecules are important components of the innate immune response to invading microorganisms and are required for migration of inflammatory and phagocytic cells to tissues where NF-κB has been activated in response to infection or injury (Bonizzi and Karin, 2004). Indirect pathways that lead to NF-κB activation are illustrated by infection of pulmonary epithelial cells with *M. tuberculosis*, which results in the release of IL-1 and activation of the classical NF-κB pathway in adjacent cells (Wickremasinghe et al., 1999).

Tumor necrosis factor-alpha (TNF-α):

TNF-α was first discovered in 1975 as a macrophage-derived molecule that induced a haemorrhagic necrosis of tumors transplanted subcutaneously in mice (Carswell et al., 1975). It is a member of the TNF superfamily of cytokines, consisting mainly of homotrimeric proteins involved with immune regulation and inflammation that includes lymphotoxin-α (TNF-β), CD40 ligand and Fas ligand (Darnay and Aggarwal, 1999). TNF-α is a pleiotropic cytokine that plays a central role in inflammation and apoptosis (Sedgwick et al., 2000; MacEwan, 2002). It is synthesized as a 26 kDa, type II transmembrane protein that is 233 amino acids

in length (Pennica et al., 1984; MacEwan, 2002). It contains a 30 amino acids cytoplasmic domain, a 26 amino acids transmembrane segment, and a 177 amino acids extracellular region (Wang et al., 1985; Ishisaka et al., 1999). TNF-α is assembled intracellularly to form a transmembrane, non-covalently-linked homotrimeric protein. The 157 amino acids residue soluble form of TNF-α (sTNF-α) is released from the C-terminus of the transmembrane protein through the activity of TNF-α-converting enzyme (TACE), a membrane-bound disintegrin metalloproteinase (Moss et al., 1997; Kriegler et al., 1988).

The granulomatous immune response is characterized by delayed hypersensitivity and is mediated by various cytokines released by the stimulated mononuclear phagocytes, including TNF-α (Morimoto et al., 1989; Ellner and Wallis, 1989). TNF-α is a pro-inflammatory cytokine and following appropriate stimulation, many cells produce TNF-α. The main cellular sources of TNF-α during an inflammatory response are monocytes (Frankenberger et al., 1996) and macrophages (Sakurai et al., 1985), but it can also be produced by other cells including NK cells, T and B lymphocytes (Ware et al., 1992), mast cells (Bissonnette et al., 1995), dendritic cells (Zhou and Tedder, 1995), astrocytes (Lee et al., 1993), osteoblasts (Modrowski et al., 1995) and neurons (Tchelingerian et al., 1996). Expression of the TNF-α gene in macrophages can be stimulated by both exogenous and endogenous factors: bacteria, viruses, parasitic organisms, irradiation, trauma, ischemia, cytokines [IL-1β, IL-2, IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF)] and by TNF-α itself (Larche et al., 2005). Lipopolysaccharide (LPS) and bacterial products are strong stimuli of TNF-α synthesis (Bazzoni and Beutler, 1996). TNF-α is recognized as a significant mediator of macrophage activation for intracellular killing of bacilli following ingestion by phagocytic cells (Wallis et al., 1993). Evidence is accumulating that TNF-α is vital to host defense and antibacterial resistance against infections caused by facultative intracellular

organisms, particularly by *M. tuberculosis* which is a potent inducer of cytokine production (Valone et al., 1988; Takashima et al., 1990).

Although this cytokine is involved in multiple cell regulatory and differentiation processes leading to immunity in tuberculous infection, however, TNF-α also mediates effects deleterious to the host contributing to the pathophysiology of tuberculosis. High levels of the cytokine at the site of infection induce an excessive damaging inflammatory response that overwhelms its beneficial effects (Bekker et al., 2000). Therefore, an understanding of the mechanisms that regulate TNF-α expression is important in achieving a harmonious balance between the outcome of both its beneficial and pathologic effects.

(A) Ligand structure and receptors:

The crystal structure of TNF indicates that it is biologically active as a trimer, both as a soluble and as a transmembrane factor (McWhirter et al., 1999; Idriss and Naismith, 2000). The type II transmembrane protein TNF can be considered as the prototype of the TNF cytokine superfamily, a core group of structurally related ligands [encompassing lymphotoxin (LT)α, LTβ, and LIGHT] (Locksley et al., 2001; Granger and Ware, 2001). The TNF cytokine family members interact with more than one receptor of the corresponding superfamily of cognate receptors. These cytokines and receptors play important roles in the coordinated development of the immune system and the protection from pathogens (Pfeffer, 2003).

Both the homotrimer TNF receptors, TNFR-I (TNFRp55: 55 kDa) and TNFR-II (TNFRp75: 75 kDa) interact with, and are involved in binding and signal transduction to both soluble and transmembrane TNF (Ehlers, 2003). However, the membrane-associated form mostly binds to TNFR-II, whereas soluble TNF preferentially binds to TNFR-I, with greater functional consequences than the former (Papadakis and Targan, 2000). TNFR-I contains a characteristic structural cassette, termed death domain, in its intracytoplasmic sequence that is conserved within a distinct subset of other TNFR family members, such as CD95, DR3, DR4 and DR5 (Tartaglia et al., 1993; Locksley et al., 2001).

(B) TNF-α signal transduction machinery:

TNFR-I has been shown to be essential for surviving infections with intracellular bacteria, such as *M. tuberculosis, Salmonella typhimurium, M. avium, Listeria monocytogenes* (Pfeffer et al., 1993; Everest et al., 1998; Ehlers et al., 1999). The death domain is required for the signal transduction of the functions of TNFR-I *in vivo* (Plitz et al., 1999). TNF-α exerts its functions by interaction with the death domain-containing TNFR-I and the non-death domain-containing TNFR-II. Important proteins that interact directly or indirectly with the cytoplasmic domains of TNFR-I and TNFR-II are receptor-interacting protein (RIP), a serine/threonine kinase, and TNF receptor-associated factor (TRAF)-1 and -2 (Orlinick and Chao, 1998; Verrecchia and Mauviel, 2004). RIP is required for NF-κB activation in response to TNF-α but not IL-1 or LPS (Kelliher et al., 1998). TRAF-1 and -2 define a novel group of adaptor proteins involved in signal transduction by most members of the TNF receptor family, of IL-1 receptor and IL-17 receptor, as well as some members of the TLRs family (Verrecchia and Mauviel, 2004). TRAF-2 is currently the best-characterized TRAF family member, having a key role in mediating TNFR1-induced signalling cascades leading to activation of NF-κB and JNK (c-Jun N-terminal kinase) (Wajant and Scheurich, 2001; Chen and Goeddel, 2002).

(1) NF-κB activation pathway:

NF-κB proteins are a family of cytoplasmic heterodimeric transcription factors that are inactive due to their association with an inhibitory protein IκB (Verrecchia and Mauviel, 2004). Potent activators, such as TNF-α, IL-1, or LPS, activate IKK complex (Bonizzi and Karin, 2004). Once activated, IKK induces rapid degradation of the IκBs (especially IκBα) within minutes. For IκBα this degradation process consists of a series of well-characterized steps, which seem to be relevant to the other IκBs (Baldwin, 1996; Ghosh et al., 1998). Inducible IκB

phosphorylation, one of the earliest events in the common activation pathway, occurs at serines 32 and 36 in IκBα, and mutation of either serine (even to a threonine) greatly inhibits the degradation process (Traenckner et al., 1995; DiDonato et al., 1996). Phosphorylation leads to the immediate recognition of IκBα by the recently identified F-box/WD40 E3RS^{IκB}/β-TrCP (Yaron et al., 1998; Laney and Hochstrasser, 1999; Maniatis, 1999), which consequently results in the polyubiquitinylation of IκBα primarily at lysines 21 and 22 by an SKp1–Cullin–F-box (SCF)-type E3 (Scherer et al., 1995; Baldi et al., 1996). This modification then targets IκBα for rapid degradation by the 26S proteasome.

The degradation of its inhibitor exposes the nuclear localization sequence (NLS) of NF-κB resulting in binding to karyopherins and translocation of NF-κB to the nucleus (Karin and Ben-Neriah, 2000). It is important that inhibitors of the 26S proteasome efficiently block NF-κB nuclear translocation, indicating that neither phosphorylation nor ubiquitinylation is sufficient to dissociate IκB from NF-κB (DiDonato et al., 1995; Alkalay et al., 1995).

TNF-α blockade: Anti-TNF-α agents:

Attenuation of the biological activity of TNF has lately become an important therapeutic intervention in the management of a wide variety of chronic inflammatory diseases (Shanahan and St Clair, 2002). Currently two major protocols are being used for the treatment of various chronic inflammatory diseases: Infliximab, an anti-TNF-α neutralizing antibody, and Etanercept, a recombinant molecule made up of two identical chains of the human 75 kDa TNF receptor II fused to the Fc portion of human IgG1 (Gardam et al., 2003).

Infliximab is a human-murine (25% murine) chimeric monoclonal antibody with high binding affinity (Bekker et al., 2000) and specificity for TNF-α (Knight et al., 1993). It forms stable complexes with the monomeric and trimeric forms of soluble TNF-α and with the transmembrane forms of TNF-α (Scallon et al., 2002). It also has the ability to cross-link TNF-α molecules. Binding to

transmembrane TNF-α results in macrophage and monocyte lysis by cytotoxicity that depends on antibodies and complement (Lugering et al., 2001). Infliximab does not bind to related cytokines, such as TNF-β, which is involved in the Th1 response (Falcone et al., 1994).

There are subtle differences in the mechanisms of action of these two agents; in particular, drug-mediated apoptosis and monocytopenia appear to be unique to infliximab (Gardam et al., 2003). Infliximab binds more avidly than etanercept does to transmembrane TNF-α and form a more stable complex. Infliximab is more effective at inhibiting transmembrane TNF-α-mediated activation of endothelial cells. Infliximab binds both the monomeric and trimeric form of soluble TNF-α, whereas etanercept effectively binds only to the trimeric form. Etanercept-TNF-α complexes are unstable, resulting in the release of soluble TNF-α. On the basis of these differences, infliximab might be predicted to have a more significant effect on the host's ability to suppress *M. tuberculosis* infection (Gardam et al., 2003).

CORRELATION BETWEEN TUBERCULOSIS AND AUTOIMMUNE DISEASES:

Like HIV-tuberculosis, SLE –TB association commonly exists as shown by various studies. The association of SLE with tuberculosis (TB) was studied in a group of 388 patients with SLE monitored between 1953-1994. TB was diagnosed in 14 patients (3.6%). The occurrence of septic fevers in SLE patients that did not respond to glucocorticoid therapy indicated the possibility of complication with TB (Rovensky et al., 1996). In a retrospective analysis of 146 systemic lupus erythbematosus (SLE) patients seen over a 5 year period, 17 patients of tuberculosis (TB) were identified yielding a prevalence rate of 11.6% (Balakrishnan et al., 1998). Results of the 526 SLE patients, 57 (11%) had TB. There is a high prevalence of TB, especially extra-pulmonary diseases, among SLE patients. High cumulative dose of corticosteroid and lupus nephritis are

important risk factors for the development of TB (Tam et al., 2002). Of the 556 patients evaluated, 20 patients (3.6%) with TB were identified. An increased frequency of TB infection and a high prevalence of extrapulmonary TB were observed in a large cohort of SLE patients. The mean daily dose of prednisolone before the diagnosis of TB and the cumulative dose of prednisolone, which possibly related to disease severity, were important determinants for the increased risk of TB in these patients with SLE (Sayarlioglu et al., 2004). These results might help to interpret the magnitude of the problem attributable to the introduction of new therapies in RA (Carmona L, et al., 2003). Tuberculosis was documented in 15 SLE and 7 RA patients with an incidence rate of 7.9/1,000 patient-years and 2.3/1,000 patient-years, respectively (p=0.003).

Conclusion: Taken together, the characteristics of tuberculosis in autoimmune disorders patients were:

(1) higher incidence rate with SLE patients,
(2) more frequent extra-pulmonary involvement,
(3) more extensive pulmonary involvement, and
(4) higher relapse rate than in rheumatoid arthritis.

Thus, the contributory role of *M. tuberculosis* infection in the morbidity and mortality of patients with SLE must be emphasized, especially in areas in which this bacterium is endemic (Yun et al., 2002). In long-term corticosteroid, 21 of these patients developed active tuberculosis. The incidence of active tuberculosis is increased in rheumatic patients on moderate-to-high dose steroid treatment (Kim et al., 1998).

INFLAMMATORY MARKERS IN AUTO IMMUNE DISORDERS

Cytokines are soluble proteins produced by a wide variety of hematopoietic and nonhematopoietic cell types. They are critical for both normal innate and adaptive

immune responses, and their expression may be perturbed in most immune, inflammatory, and infectious disease states.

Cytokines are involved in the regulation of the growth, development, and activation of immune system cells and in the mediation of the inflammatory response. In general, cytokines are characterized by considerable redundancy in that different cytokines have similar functions. In addition, many cytokines are pleiotropic in that they are capable of acting on many different cell types. This pleiotropism results from the expression on multiple cell types of receptors for the same cytokine, leading to the formation of "cytokine networks". The action of cytokines may be:

(1) autocrine when the target cell is the same cell that secretes the cytokine,

(2) paracrine when the target cell is nearby, and

(3) endocrine when the cytokine is secreted into the circulation and acts *distal to the source.*

THE TUMOR NECROSIS FACTOR LIGAND AND RECEPTOR FAMILIES

Tumor necrosis factor (TNF-α) and lymphotoxin-α were isolated more than 10 years ago, on the basis of their ability to kill tumor cells in vitro and to cause hemorrhagic necrosis of transplantable tumors in mice (Carswell et al., 1975). The complementary DNAs and genes encoding each protein were cloned immediately thereafter (Pennica et al., 1984; Aggarwal et al., 1984). Concurrently, a factor known as cachectin was isolated from mouse macrophages, sequenced, and shown to be identical to TNF-α (Beutler et al., 1985). Cachectin was identified not as a cytolysin, but as a catabolic hormone that suppressed the expression of lipoprotein lipase and other anabolic enzymes (Kawakami et al., 1982; Pekala et al., 1984; Torti et al., 1985). Still other studies demonstrated the powerful pro-inflammatory effects of TNF-α (Dayer et al., 1985; Gamble et al., 1985) and revealed its role as a central endogenous mediator of

endotoxic shock (Beutler et al., 1985; Tracey et al., 1986). Hence, TNF-α has a broad spectrum of biologic activities.

Because it proved to be highly toxic in animals and humans, TNF-α did not fulfill initial expectations that it would be useful in the treatment of cancer. However, considerable evidence suggests that overproduction or inappropriate production of TNF-α may play a part in various chronic inflammatory diseases. Produced largely by macrophages in response to inflammatory stimuli such as lipopolysaccharide, TNF-α binds to receptors present on virtually all cells throughout the body. TNF-α, if released systemically in large amounts all at once, modifies the anticoagulant properties of endothelial cells, activates neutrophils, and induces the release of other inflammatory cytokines. These effects culminate in cardiovascular collapse. By contrast, chronic, low-level production of TNF-α may contribute to the inflammatory response. Bone resorption, fever, anemia, and wasting may all, in some measure, be attributable to TNF-α (Beutler et al., 1992). TNF-α is one of 10 known members of a family of ligands that activate a corresponding family of structurally related receptors.

The receptors initiate signals for cell proliferation and apoptosis (programmed cell death). These signals are required for the normal development and function of the immune system. Excessive signaling through some of the receptors can cause severe inflammatory reactions, tissue injury, and shock. Mutations of the genes corresponding to the ligands or the receptors can cause characteristic disturbances of lymphocytes, derangement of the immune response, or autoimmune disease.

All members of the TNF- ligand family are believed to consist of three polypeptide chains. All but lymphotoxin-β (which consists of a single lymphotoxin-α subunit and two lymphotoxin-β subunits) are made up of three identical subunits. All except lymphotoxin-α (which is entirely secreted) and TNF-α (which is predominantly secreted) are transmembrane proteins that act chiefly through cell-to-cell contact. Nerve growth factor, a dimeric protein, is not actually a member of

the TNF-ligand family. Rather, it was apparently adapted in the course of evolution to serve its receptor, a true member of the TNF-α receptor family.

All members of the TNF-α receptor family are believed to be transmembrane proteins that consist of two identical subunits. The family is defined by a cysteine-rich amino-acid motif that recurs three to six times in the extracellular domain. The cytoplasmic domains vary more than the extracellular domains. Notably, certain receptors contain a 60-residue cytoplasmic sequence known as the "death domain." In the 55-kd TNF-α receptor and the Fas receptor, this domain is required for the transduction of an apoptotic signal.

With the exceptions of TNF-α and lymphotoxin-α each member of the ligand family binds to a specific receptor. TNF-α and lymphotoxin-α engage two receptors (the 55-kd and 75-kd TNF-α receptors) with similar affinity. These two cytokines initiate similar (if not identical) biologic responses, although they are produced by different types of cells (lymphotoxin-α is produced exclusively by lymphocytes and natural killer cells, and TNF-α predominantly by macrophages) in response to different stimuli (antigenic or mitogenic stimuli for lymphotoxin-α and lipopolysaccharide or other macrophage-activating agents for TNF-α).

Functions of TNF- Ligands and Receptors

For many years, the role of members of the TNF- ligand and TNF-α receptor families in immunity and in the development of the immune system remained speculative. The first indication of their function came from the finding that mutations of the ligands or their receptors could cause disease. Striking examples are the *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mutations of mice, which were found to specify defects of the Fas receptor (*lpr*) and the Fas ligand (*gld*). These mutant mice have long been taken as models of systemic lupus erythematosus because they have lymphadenopathy and splenomegaly and form autoantibodies (Izui et al., 1984; Roths et al., 1984). In the homozygous state, each mutation causes the accumulation of large numbers of T cells lacking the CD4 and CD8 surface

proteins. Heterozygous pairing of the lpr^{CG} allele (CG denotes "complements *gld*") with the *gld* allele also causes lymphoproliferation, suggesting that the products of the two loci might interact with one another (Matsuzawa et al., 1990).

Further study demonstrated that the lpr^{CG} allele encodes a mutant Fas receptor that can bind its ligand but lacks signal-transducing activity. The classic *lpr* mutation, by contrast, abolishes the expression of the Fas receptor (Allen et al., 1990; Watanabe-Fukunaga et al., 1992; Adachi et al., 1993). The lpr^{CG} allele contains a point mutation within the death domain of the Fas receptor that prevents signal transduction but does not affect ligand binding (Watanabe-Fukunaga et al., 1992). The *gld* allele contains a point mutation that inactivates the Fas ligand (Suda et al., 1993). It is widely suspected that mutations of the Fas ligand or its receptor cause a failure of apoptosis in T lymphocytes. This, in turn, leads to massive accumulations of lymphocytes in lymph nodes and spleen and features of autoimmunity.

Other members of the TNF- ligand and TNF-α receptor families are also important in immune function. For example, the syndrome of X-linked immunodeficiency, in which there are high levels of IgM and low or absent levels of other immunoglobulins, is caused by a mutation in the CD40 ligand (Allen et al., 1993; Korthäuer et al., 1993). Interaction of the CD40 ligand on T cells with the CD40 receptor on B cells mediates immunoglobulin-class switching (the conversion from the production of IgM antibodies to the production of IgG antibodies) and clonal expansion of antigen-responsive B cells. In mice, deletion of the CD40-ligand or CD40-receptor genes results in a phenotype that resembles the disease that occurs in humans (Xu et al., 1994; Kawabe et al., 1994).

How the Receptors Work

Interactions between TNF-α and its receptors are presumably typical of interactions between the other ligand–receptor pairs in these two molecular families. Trimeric ligands of the TNF-α family may cause aggregation or

clustering of receptor subunits, thereby triggering a cellular response, since antibodies against either of the two TNF-α receptors mimic the actions of TNF-α (Engelmann et al., 1990; Tartaglia et al., 1993). Support for the aggregation model is bolstered by the crystal structure of lymphotoxin-α, which forms a complex with extracellular-domain fragments of the 55-kd TNF receptor, (Banner et al., 1993) in which three receptor fragments crystallize with each lymphotoxin-α trimer.

One of the most intriguing actions of TNF-α is the induction of apoptosis. Apoptosis is almost certainly relevant to some of the toxic effects of TNF-α, such as shock and inflammation. TNF-α induced apoptosis may also have physiologic relevance, as does the apoptosis induced by the Fas receptor. It is possible, for example, that TNF-α mediated apoptosis of infected cells helps protect the host. It is likely that both TNF-α receptors participate in cell death, although the 55-kd receptor is more potent than the 75-kd receptor. Since the cytoplasmic domains of the two receptors are structurally different, each must initiate apoptosis through distinct mechanisms.

An entirely different class of cytoplasmic proteins binds to the 55-kd TNF-α receptor and the Fas receptor. These proteins are important in transducing signals for programmed cell death. The Fas-associated death domain (FADD), (Chinnaiyan et al., 1995) also called MORT-1, (Boldin et al., 1995) the TNF-α receptor–associated death domain (TRADD), (Hsu et al., 1995) and the receptor interacting protein (RIP) (Stanger et al., 1995) bind to the Fas receptor, the 55-kd TNF-α receptor, and both receptors, respectively. Each of these proteins contains a version of the death domain found within the receptors themselves. This motif permits interaction between receptor and transducer molecules.

FADD (Boldin et al., 1995; Chinnaiyan et al., 1995) is a proximal transducer of the apoptotic activity of the Fas receptor, with which it forms a heterodimer. Engagement of the ligand causes the release of homodimeric FADD, which relays the death signal to the cytoplasm. FADD is incapable of forming

heterodimers with receptors encoded by the mutant *lpr*^{CG} gene. Hence, it is at precisely this level that the *lpr*^{CG} mutation interrupts signaling.

Acting in an analogous fashion, TRADD is a proximal transducer of apoptosis mediated through the 55-kd TNF-α receptor (Hsu et al., 1995). RIP appears to serve both the 55-kd TNF-α receptor and the Fas receptor. The transducers may interact with distinct targets downstream from the receptor. Although overexpression of any of the transducers can initiate cell death, different portions of each protein act to carry the apoptotic signal forward within the cytoplasm (Cleveland et al., 1995). Moreover, RIP is far larger than either TRADD or FADD and contains a kinase domain (Cleveland et al., 1995).

In accordance with the molecular-switch hypothesis, a receptor modified by the binding of ligand might effectively catalyze the formation of homodimeric FADD, TRADD, or RIP. Causing conformational changes in these molecules that lead to further reactions in the signaling cascade. This scenario is consistent with the observation that modified receptors are constitutively activated in the absence of ligand (Bazzoni et al., 1995).

CORRELATION OF TNF-α AND AUTOIMMUNE DISEASES

Low levels of TNF-α may account for the state of insulin resistance that contributes to the development of type-1 diabetes mellitus (Hotamisligil et al., 1995; Hofmann et al., 1994; Hotamisligil et al., 1994; Hotamisligil et al., 1993; Spiegelman et al., 1993). The lupus-like state that follows abrogation of the function of the Fas ligand or receptor suggests that some autoimmune disorders could involve defects in the Fas- or TNF-α signaling axes. The observation that the administration of TNF-α attenuates or prevents some autoimmune diseases in animals supports this view. (Jacob et al., 1990; Yang et al., 1994; Yang et al., 1998). Both TNF-α and interleukin-1 are likely to have primary roles in the

pathogenesis of rheumatoid arthritis. The serum and synovial concentrations of both cytokines are high in patients with active rheumatoid arthritis.

CORRELATION OF TNF-α AND TUBERCULOSIS

Initiation of TNF-α clearly predisposes to certain infections, such as granulomatous infection like Tuberculosis (Islam et al., 2004; Wilkinson et al., 2001). A definite correlation exists between nitric oxide release and TNF-alpha production, irrespective of low or high production in MDR-TB or fresh cases, respectively. The present data suggest that peripheral blood monocytes of MDR-TB patients typically show signs of immunosuppression. Whether such immunodepression is the cause or the effect of MDR-TB merits further investigation (Sharma et al., 2004). Data suggest that the PBMC of MDR-TB patients typically show TNF-alpha depression in response to the 30-kDa antigens, and this effect is modulated by IL-10. In addition, we highlight the role of TNF-alpha in IL-8 secretion in MDR-TB patients. (Lee et al., 2003). Findings may indicate that, during TB, predisposition of CD4 T-cells to apoptosis may involve both low expression of Bcl-2, and excessive expression of TGF-beta, TNF-alpha and Fas-L. (Hirsch et al., 2005). The serum TNF-α, soluble CD8 (sCD8) and soluble interleukin-2 receptor (SIL-2R) levels were probed in children with active pulmonary tuberculosis (n=66) and healthy controls (n=20). Measurable serum TNF-α levels were detected in nine of 86 children (10.5%), all of who belonged to the group with active disease. Serum sCD8 and SIL-2R determinations revealed a significant difference between the group with active pulmonary tuberculosis and the controls ($p<0.05$) (Barlan et al., 1995). Findings suggest that the underlying mechanisms of cytokine regulation might differ between N-TB and R-TB patients, and that decreased IL-12 production in response to the 30-kDa or PPD Ag might be involved in the immunopathogenesis of human R-TB. (Lee et al., 2002). The increases of TNF-alpha and IL-12 level were observed in comparison to control group. TNF-alpha concentration was about 2-fold higher in the positive patients than it was in control group; IL-12

concentration was about 4-fold higher and the differences between IL-12 levels were statistically important ($p<0.05$). However, no significant differences were found in IFN-gamma level among all groups. Using Spearman correlation rank test, a significant correlation was found between TNF-alpha and IL-12 in the positive patient group. The correlation factor was more significant for the group of patients with *Mycobacterium tuberculosis* present in blood and urine than it was in urine positive PCR group ($r= -0.66$ vs. $r= -0.51$) (Koziol-Montewka et al., 2004).

OXIDATIVELY MODIFIED AUTOANTIGENS IN AUTOIMMUNE DISEASES

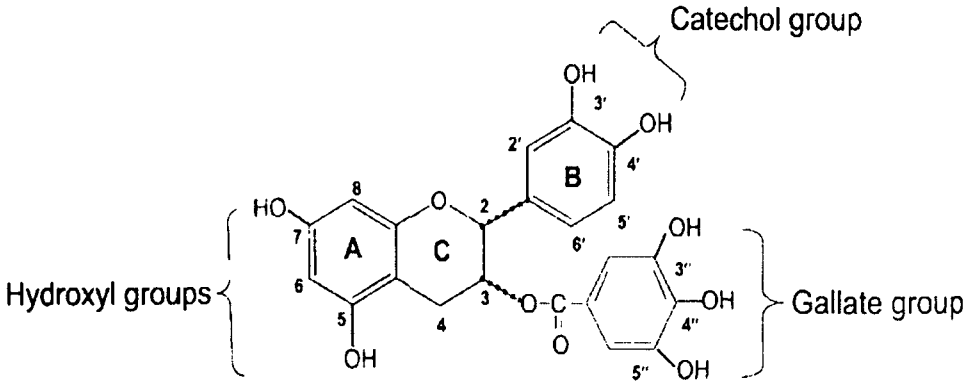
Reports indicate (Kurien et al., 2006) that free radical-mediated oxidative damage and consequent protein modification by the end products of oxidative damage are important mediators of cell toxicity and disease pathogenesis. Aldehydic products, mainly the 4-hydroxy-2-alkenals, form adducts with proteins and make them highly immunogenic. Oxidative modification of proteins has been shown to elicit antibodies in a variety of diseases including systemic lupus erythematosus (SLE), alcoholic liver disease, diabetes mellitus (DM), and rheumatoid arthritis (RA). Oxidatively modified DNA (8-oxodeoxyguanine) and low-density lipoproteins (LDL) occur in SLE, a disease in which premature atherosclerosis is a serious problem. In addition, it has been reported that immunization with 4-hydroxy-2-nonenal (HNE)-modified 60-kDa Ro autoantigen elicits an accelerated epitope spreading in an animal model of SLE. Advanced glycation end product (AGE) pentosidine and AGE-modified IgG have been shown to correlate with RA disease activity. Oxidatively modified glutamic acid decarboxylase is important in type 1 DM, while autoantibodies against oxidized LDL are prevalent in Behcet's disease. The fragmentation of scleroderma-specific autoantigens occurs as a result of oxidative modification and is thought to be responsible for the production of autoantibodies through the release of cryptic epitopes. In the face of overwhelming evidence for the involvement of oxidative damage in autoimmunity the administration of antioxidants is a viable

untried alternative for preventing or ameliorating autoimmune disease, although results in cardiovascular disease were disappointing (Kurien et al., 2006). Furthermore, free radical mediated peroxidative damage resulting in augmented MDA levels in systemic lupus erythematosus patients has been well established (Kurien and Scofield, 2003)

Natural antioxidant in disease prevention – EGCG (Epigallocatechin-3-gallate) from Green Tea:

Tea (*Camellia sinensis*) has been used as a daily beverage for several thousand years since it was introduced as a beverage in China that is now the second most common beverage consumed by humans (Weisburger, 1999). Even though it has been known traditionally that tea may have some beneficial health effects, such effects were not demonstrated by well-controlled laboratory studies until the 1970s (Weisburger, 1999). However, recent studies have revealed the biological effects of tea, such as antitumor as well as antimicrobial effects, even at the molecular level. The active components of tea responsible for such biological effects are now known to be catechins (also known as polyphenols), which constitute seven forms, including epigallocatechin gallate (EGCG). EGCG is a major catechin compound in tea extracts and is also the most active form among the tea catechins in a variety of biological activities. For instance, EGCG has anticarciogenic (Katiyar et al., 1993; Yamane. et al., 1995), antioxidant (Ho et al., 1992), as well as antimicrobial activities (Yam et al., 1997; Yam et al., 1998). Although the mechanism of antimicrobial activity of EGCG has been studied, it is still unclear till date. However, the immunomodulatory effect of EGCG has been increasingly recognized, since the bioavailability of EGCG in plasma after drinking tea is known to be high (Yang et al., 1998). In fact, it is known that EGCG potently stimulates the production of interleukin-1 alpha (IL-1a), IL-1b, and tumor necrosis factor alpha (TNF-α) by cultured human peripheral blood mononuclear cells (Katiyar, S. et al., 1999). Furthermore, EGCG protects

against UV radiation-induced immunosuppression and tolerance induction by reducing IL-10 production and increasing IL-12 production in epidermal and dermal cells (Sakagami et al., 1995). However, the detailed immunomodulatory effects of EGCG on immune cells have not been investigated. The chemical structure of EGCG is given below.



Objectives of the present study:

Thus, in the present study, investigations were carried out on an appreciable number of SLE autoimmune disorder patients for immunological evaluation of *Mycobacterium tuberculosis* infection admitted or attending Medical OPD's of J. N. Medical College Hospital. The study was carried out to achieve the following objectives: (a) to probe the incidence for clinical characteristics of *Mycobacterium tuberculosis* infection in patients with SLE autoimmune disorder, (b) to evaluate the effect of *Mycobacterium tuberculosis* infection on the clinical course of morbidity, mortality of autoimmune disorders and vice-versa and (c) to correlate various immuno-inflammatory markers with disease activity in Tuberculosis associated autoimmune diseases. Briefly, in this investigation, a systematic study was carried out using the techniques of real-time RT-PCR, direct binding ELISA and competition ELISA to characterize the sera and monocytes of patients with SLE, TB and SLE-TB with respect to TNF-α. The modulatory effects of the natural antioxidant EGCG and NAC on monocyte cultures of SLE, TB and SLE-

TB patients were evaluated at the protein level as well as immunologically. To fully delineate the contribution of the innate immune response to the pathogenesis of the above diseased patients, the roles played by ROIs and RNIs, induction of TNF-α, activation of NF-κB and monocyte activation were examined and correlated by immunological investigations.

Toxicity assessment of EGCG on viability of human monocytes was assessed by cell viability assays like MTT and trypan blue exclusion assay as well as by assessing human housekeeping genes like 18S rRNA by real-time RT-PCR, respectively. RT-PCR, real-time RT-PCR and ELISA probed the effects of allicin on expression of TNF-α and M. tuberculosis 85B in M. tuberculosis-infected monocytes after 24 hours of infection. Commercially available ELISA kits assessed the repertoire of TNF-α in supernatants of monocyte cultures from patients and healthy controls that were treated and untreated with EGCG. Glutathione peroxidase activities as well as MDA levels due to lipid peroxidation were evaluated in patient's monocytes in the presence or absence of EGCG, and in turn, were correlated with binding studies in immunological investigations.

It is hoped that the data generated in the present study may help in the better understanding about the etiology and trigger / autoantigen contributing towards SLE.

MATERIALS & METHODS

MATERIALS AND METHODS:

Materials

Monoclonal anti-TNF-α antibody, N-acetyl-cysteine (NAC), SN50 (an inhibitor of NF-κB) and its analogue SN50/M, Epigallo-catachin-3-gallaterum, Bovine Serum Albumin, Coomassie Brilliant Blue G-250 and R-250, p-nitrophenyl phosphate, anti-human IgG alkaline phosphate conjugate, Tween-20, dithiothreiotol, phenylmethylsulphonyl fluoride, standard protein markers, protein A sepharose CL-4B, sodium azide, ethidium bromide, chloroform, isoamyl alcohol, reduced glutathione, glutathione reductase, cumene hydroperoxide and reduced β-nicotinamide adenine dinucleotide phosphate were from Sigma Chemical Company, U.S.A. SN50 is a hybrid peptide containing nuclear localization sequence of *p50 subunit of NF-κB heterodimer and has been shown to* completely inhibit the translocation of NF-κB in human cell lines at 100 μg/ml (Lin et al., 1995).

Ficoll-Paque was from Pharmacia, Piscataway, NJ, U.S.A. Middlebrook 7H9 broth, Middlebrook ADC enrichment fluid, RPMI-1640 medium were from HiMedia, India. MTT cell viability assay kit and immunoassay kits for TNF-α were of R & D Systems, U.S.A. 12-wells tissue culture plates were obtained from Techno Plastic Products (TPP), Switzerland. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were from NUNC, Denmark. A colorigenic substrate p-nitrophenyl phosphate was obtained from C.S.I.R. Centre for Biochemicals, New Delhi. Asparagine, citric acid, magnesium sulphate, dipotassium hydrogen phosphate, ferric ammonium citrate and glycerol were obtained from Qualigens, India. Sodium pyruvate was obtained from SRL, India. All other chemicals were of highest grade available.

Study subjects:

For isolation of peripheral blood mononuclear cells (PBMC's) or serum, venous blood was obtained from patients with systemic lupus erythematosus (SLE),

tuberculosis (TB) and SLE-TB admitted or attending O.P.D. at J.N. Medical College Hospital of A.M.U, Aligarh. Also, for some experiments involving *in vitro* infection with pathogenic strain of MTB for using the infected cells as valuable antigens / inhibitors in immunoassays, we obtained healthy nonsmoking adult volunteers with no history of tuberculosis or positive tuberculin skin test. The SLE sera showed high titer anti-DNA antibodies and fulfilled the American College of Rheumatology (formerly American Rheumatism Association) revised criteria for the classification of SLE (Arnett et al., 1988). Serum samples were decomplemented by heating at 56°C for thirty minutes and stored in small aliquots at -20°C until use with 0.1 percent sodium azide as preservative.

Exclusion criteria

Patients who have already received steroids/cyclophosphamide and other immunosuppressant as well as patients having hepatitis, septicemia, multi organ failure, were excluded from the study.

Methods

Determination of protein concentration:

Protein was estimated by the methods of Lowry et al. (1951) and Bradford (1976) (data not shown).

(A) Protein estimation by the Lowry (Folin-Ciocalteau) method:

Protein estimation by this method involves complexing of the protein's peptide bonds with Cu^{2+} under alkaline conditions (Lowry et al., 1951). The resultant Cu^{+} appears to catalyze the oxidation of tyrosine and tryptophan residues by reducing phosphomolybdotungstate anions in the Folin reagent (a mixture of sodium tungstate, molybdate and phosphate), added subsequently. This reaction develops a blue colour due to the formation of heteropolymoiybdenum blue, which can be quantified by its absorbance at 660 nm.

Reagents:

(i) Folin-Ciocalteau reagent

The reagent was diluted 1:4 with distilled water before use.

(ii) Alkaline copper reagent

The components of alkaline copper reagent were prepared as follows:

(a) 2% sodium carbonate in 100 mM NaOH

(b) 0.5% copper sulphate in 1% sodium potassium tartarate

The working reagent was prepared fresh before use by mixing the two components in the ratio 50:1, respectively.

Procedure:

To 1.0 ml of protein sample was added 5.0 ml of freshly prepared alkaline copper reagent. After thorough mixing, the reaction mixture was allowed to stand at room temperature for 10 minutes, followed by the addition of 1.0 ml of 1:4 times diluted Folin-Ciocalteau reagent. The contents were mixed immediately. The reaction was allowed to proceed for 30 minutes at room temperature and each tube was subsequently monitored at 660 nm. The protein content of the unknown sample was determined by using bovine serum albumin to construct a standard calibration curve.

(B) Protein estimation by the Bradford method:

This method is based on strong binding of the dye Coomassie Brilliant Blue G-250, in acidic medium, to protein hydrophobically and at positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color develops (λ_{max}-595 nm).

Preparation of dye:

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1.0 litre and filtered through Whatman No. 1 filter paper to remove undissolved particles.

Procedure:

To 1.0 ml of solution containing 10–100 µg protein was added 5.0 ml of dye solution. The contents were mixed thoroughly by vortexing. The absorbance was read at 595 nm after 5 minutes against a reagent blank.

Polyacrylamide gel electrophoresis of proteins:

Polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970) (data not shown as serum samples from patients were analyzed in comparison to normal controls for preliminary experiments).

Reagents:

(i) Acrylamide-bisacrylamide (30:0.8)

A stock solution of 30% acrylamide containing 0.8% bisacrylamide was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in an amber coloured bottle.

(ii) Resolving gel buffer

A stock solution was prepared by dissolving 36.3 gm Tris base in 48 ml of 1 N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

(iii) Stacking gel buffer

6.05 gm Tris was dissolved in 40 ml distilled water, pH adjusted to 6.8 with 1 N HCl and the final volume adjusted to 100 ml with distilled water.

(iv) Electrode buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and the final volume made up to 1.0 litre with distilled water.

(v) Sample buffer

(a) 6.0 gm of Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid. The final volume was brought to 100 ml with distilled water.

(b) 1.0 mg of bromophenol blue and 12.5 ml of glycerol were added to 12.5 ml of the above solution. β-mercaptoethanol was added just before use.

Recipe for 10–20% Gradient Gel

Reagents	**10%**	**20%**
Acrylamide-bisacrylamide (30:0.8)	5.0 ml	10 ml
Resolving gel buffer	3.8 ml	3.8 ml
10% SDS	150 µl	150 µl
10% Ammonium persulphate	50 µl	50 µl
TEMED	10 µl	10 µl

Resolving Gel (total volume: 30 ml)

Reagents	**10%**	**20%**
Acrylamide-bisacrylamide (30:0.8)	5.0 ml	10 ml
Resolving gel buffer	3.8 ml	3.8 ml
10% SDS	150 µl	150 µl
10% Ammonium persulphate	50 µl	50 µl
TEMED	10 µl	10 µl

The final volume was raised to 15 ml each with distilled water.

2.5% Stacking Gel (total volume: 10 ml)

Acrylamide-bisacrylamide (30:0.8)	0.8 ml
Stacking gel buffer	2.5 ml
10% SDS	100 µl
10% Ammonium persulphate	75 µl
TEMED	25 µl

The final volume was raised to 10 ml with distilled water.

Recipe for 7.5% SDS-PAGE (total volume: 10 ml)

Acrylamide-bisacrylamide (30:0.8)	2.5 ml
Resolving gel buffer	2.5 ml
10% SDS	100 µl
10% Ammonium persulphate	50 µl
TEMED	10 µl

The final volume was raised to 10 ml with distilled water.

Procedure:

The glass plates (18 cm x 16 cm) were soaked in chromic acid and thoroughly washed with tap water followed by a final rinse with distilled water and ethanol. The plates were dried and sealed with 1% agarose and 1.5 mm thick spacers. The reagents were mixed and poured between the glass plates. The resolving gel was allowed to polymerize at room temperature, following which, the stacking gel was layered on top. A well-forming comb was inserted immediately and the gel was left to polymerize at room temperature. In case of gradient gels, a gradient of resolving gel was formed with the help of a gradient former (Bio-Rad, model 385). After ensuring complete polymerization, the protein samples (25–100 µg) in one-fourth volume of sample buffer were electrophoresed at 80 volts

at room temperature. The gels were stained using 0.25% Coomassie Brilliant Blue R-250 or with silver stain reagent.

Silver staining:

Silver staining was done by the method of Merril et al. (1983). Briefly, the gel was incubated in 40% methanol and 12% acetic acid for 45 minutes followed by incubation in 50% ethanol for 30 minutes. Next the gel was treated with 0.02% hypo (sodium thiosulphate) for 1 minute. After washing with distilled water, the gel was placed in 0.2% silver nitrate (with 0.05% v/v formaldehyde), washed again with distilled water, and transferred to a 6% solution of sodium carbonate (with 0.05% v/v formaldehyde). After colour development, the gel was washed with distilled water and treating the gel with 3% v/v acetic acid and 5% v/v methanol arrested the reaction. All the reagents used in this procedure were freshly prepared.

Agarose gel electrophoresis:

Agarose gel was prepared by bringing 2% agarose to molten state in electrophoresis buffer (0.04 M Tris acetate, pH 8.0 containing 0.002 M EDTA). Molten agarose was poured on the gel tray and allowed to solidify for 1 hour at room temperature. The nucleic acid samples in one-tenth volume of stop-mix (30% ficoll, 0.025% xylene cyanole FF and 500 mM EDTA in 10X TAE buffer) were electropheresed for 2–4 hours at 30 mA in the same buffer. The gel was stained with ethidium bromide (0.5 µg/ml).

Isolation of IgG by affinity chromatography:

Protein A sepharose CL-4B was employed as the affinity matrix. It was swelled in 10 mM PBS, pH 7.4 at room temperature for 12 hours. The swelled matrix was washed with PBS and packed in a column having a dimension of 0.9 cm x 5 cm. The packed column was washed with 0.1 M sodium citrate, pH 3.0 in order

to elute any bound material. This was followed by equilibration of the packed matrix with 5 volumes of PBS, pH 7.4. Serum diluted with equal volume of PBS, pH 7.4 was loaded onto the column and allowed to bind at a flow rate of 20 ml/hour. Unbound protein was eliminated with PBS and absorbance of the effluent monitored till a negative absorbance was obtained at 280 nm. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride. To prevent the effect of acidic elution buffer on IgG, fractions were collected in 1 M Tris-HCl, pH 8.5. The fractions containing IgG were monitored at 280 nm. The IgG concentration was determined considering 1.4 OD_{280} = 1.0 mg IgG/ml. The isolated IgG was dialyzed against 10 mM PBS, pH 7.4 and stored at –20°C with 0.1% sodium azide. To check the purity of IgG, the samples were subjected to 7.5% SDS-PAGE.

Gel retardation assay:

The binding of TB-IgG with *M. tuberculosis* sonic extract proteins as well as with MTCF proteins was analyzed by altered electrophoretic mobility on SDS-PAGE under non-reducing conditions. *M. tuberculosis* proteins were allowed to interact with TB-IgG for 2 hours at 37°C and overnight at 4°C. This resulted in the formation of immune complex (IC). Thereafter, the complex was electrophoresed on 7% SDS-PAGE under non-reducing conditions for 2 hours at 80 V. The electrophoresed gels were visualized by staining with 0.25% Coomassie Brilliant Blue R-250.

Enzyme-linked immunosorbent assay (ELISA):

Antibodies were detected and quantified by ELISA using polystyrene flat-bottom microtitre plates as solid phase. The method described by Alam et al (1992) and Islam and Ali (1998) was followed for the assay.

Buffers and reagents:

(i) Tris-buffered saline (TBS)

10 mM Tris, 150 mM NaCl, pH 7.4

(ii) Tris-buffered saline Tween-20 (TBS-T)

20 mM Tris, 144 mM NaCl, 2.68 mM KCl and 1.0 ml/litre Tween-20, pH 7.4

(iii) Bicarbonate buffer

15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6

(iv) Substrate buffer (for anti-human IgG alkaline phosphatase conjugate)

15 mM sodium carbonate, 35 mM sodium bicarbonate and 2 mM magnesium chloride, pH 9.6

Substrate: 0.5 mg/ml of p-nitrophenyl phosphate

(A) Direct binding ELISA:

Polystyrene microtitre plates were incubated with 100 µl of protein antigen (30 µg/ml in carbonate/bicarbonate buffer, pH 9.6) for two hours at room temperature followed by overnight incubation at 4°C. The plates were washed thrice with TBS-T and unoccupied sites blocked by 150 µl of BSA (1.5% in TBS, pH 7.4) for 4–6 hours at room temperature. Serially diluted sera in TBS were added to antigen-coated as well as control (antigen uncoated) wells. The antigen-antibody interaction was allowed to proceed for two hours at room temperature followed by overnight incubation at 4°C and subsequently the plates were washed four times with TBS-T in order to remove the unbound antibodies. Bound antibodies were assayed by means of appropriate anti-immunoglobulin alkaline phosphatase conjugate using p-NPP as substrate. The reaction was stopped with 3 N NaOH and the absorbance of each well was monitored at 405 nm on an ELISA microplate reader. Each sample was coated in duplicate and the results were expressed as a mean of $A_{test} - A_{control}$.

(B) Inhibition ELISA:

The antigen binding specificity of antibody was determined by inhibition experiments (Hasan et al., 1991). Varying concentration of inhibitors (0–20

µg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated for two hours at 37°C followed by overnight incubation at 4°C. The resulting immune complex was employed in the immunoassay instead of serum. The rest of the steps were as in direct binding ELISA. The results were expressed as percent inhibition.

$$\text{Percent inhibition} = \left(1 - \frac{A_{inhibited}}{A_{uninhibited}}\right) \times 100$$

Preparation of mycobacteria:

Virulent laboratory-adapted *M. tuberculosis* (H37Rv) were grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC enrichment fluid at 37°C in 5% CO_2. Mycobacterial cultures were harvested at midlogarithmic (14 days) phase. Aliquots of the stock were kept at −70°C until use. The viability of the stock remained >99% at 1 year. Before use, aliquots were defrosted, then vortexed with glass beads for 15 minutes, followed by equilibration at 37°C for 45 minutes. This treatment results in single cell suspension of mycobacteria (Toossi et al., 1996).

Isolation of mycobacterial antigens:

Midlogarithmic mycobacterial cultures (14 days) were heat-killed at 100°C for one hour in a boiling water bath, followed by centrifugation at 10,000 rpm for 30 minutes at 4°C. The culture filtrate containing secreted proteins was separated and stored at –20°C until further use.

(A) Desalting of *M. tuberculosis* culture filtrate (MTCF) proteins:

The supernatant was sterilized by filtration through a 0.22 micron pore size membrane (Millipore Corp., USA). MTCF was concentrated 50 fold by

ammonium sulphate and dialyzed against 10 mM PBS, pH 7.4 for desalting. The protein content was determined by the method of Lowry et al. (1951) against a BSA standard. The culture filtrate preparations were stored in small aliquots at –20°C.

(B) *M. tuberculosis* sonic extract (MTSE) proteins:

Heat-killed bacilli were washed with sterile TBS, pH 7.4 and suspended in sonicating buffer containing 1 mM DTT, 1 mM PMSF, 1.0 µl/ml β-mercaptoethanol and proteinase inhibitors cocktail in TBS, pH 7.4. Sonicating at 4°C for 15 minutes in a sonicator disrupted the cell suspension and the cell debris was removed by ultra centrifugation at 20,000 rpm for 15 minutes. The protein content was determined by the method of Lowry et al. (1951). The sonic extract preparations were stored at –20°C in small aliquots until required.

Preparation of RPMI-1640 medium:

Dehydrated RPMI-1640 medium of one unit vial (16.3 gm) was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought to 1000 ml with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4°C till use.

Preparation of PBMC:

Step 1: In order to isolate peripheral blood mononuclear cells (PBMCs), 60 ml of blood was drawn from a healthy volunteer into 60 cm^3 syringes containing

3.8 units heparin/ml. The heparinized blood, in 15 ml aliquots, was transferred to sterile 50 ml polypropylene centrifuge tubes and diluted 1:1 with sterile 10 mM, PBS, pH 7.4 at room temperature, followed by gentle mixing by inverting the tube a few times.

Step 2: Diluted blood was under layered with 15 ml of Ficoll-Paque at room temperature using an 18 gauge spinal needle. Care was taken to prevent mixing of the layers. The gradient was centrifuged at 1800 rpm for 30 minutes at room temperature with the centrifuge brake turned off.

Step 3: Using a sterile pipette, the upper clear layer containing plasma was removed. The PBMCs appeared as a dense white band (buffy layer) above the red blood cells and granulocytes layer. This was removed with another sterile pipette. The banded cells were combined in 10 ml aliquots.

Step 4: Ten milliliters of banded PBMCs were diluted with 25 ml of PBS in sterile 50 ml polypropylene centrifuge tubes and centrifuged at 1100 rpm for 12 minutes at room temperature to remove platelets, which remain in the supernatant. The PBMC pellets were combined to four tubes, diluted in 30 ml PBS and centrifuged at 1100 rpm for 10 minutes at room temperature. This wash was repeated.

Step 5: The pellets were then combined and resuspended in 30 ml complete medium (CM) (RPMI-1640 medium containing 2 M L-glutamine, 25 mM HEPES, and no antibiotics). An aliquot was diluted 20-fold and counted using a hemocytometer under a light microscope using 10x ocular and 40x objective.

Preparation of autologous serum for monocyte culture:

From the same donor, 30 ml of blood was drawn without anticoagulant and transferred to serum separator tubes. The blood was allowed to clot for at least 30 minutes, then centrifuged at 3000 rpm for 15 minutes at room temperature

and the serum filtered through a sterile 0.22 µm filter unit. Autologous serum can be stored for a year or longer at –20°C.

Cell culture:

PBMCs ($5x10^6$ cells/well) were added in 12-wells tissue culture plates in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO_2 for 1–2 hours for adherence. Thereafter, non-adherent cells were removed by washing the plates extensively 4 times with RPMI-1640 medium. The adherent monocytes were cultured in RPMI-1640 supplemented with 2% autologous serum, followed by overnight resting at 37°C, 5% CO_2. This population of adherent cells is up to 95% monocytes, as observed by cytostaining and is 99% viable (Toossi et al., 1996). Prior to infection, the plates were washed twice with RPMI-1640 medium.

Infection and co-culture of monocytes with supplements:

Healthy monocytes were infected with *M. tuberculosis* ($H_{37}Rv$) at 1:1 (bacteria/cell) in 30% autologous unheated serum for 90 minutes at 37°C, 5% CO_2. Subsequent to this, the infected monocytes were washed four times with complete medium. Cells harvested at this time point were considered as time zero after infection (t_o). Other cultures received RPMI-1640 medium with 2% autologous serum. As per experimental design, some cultures, immediately after infection received 10 ng/ml anti-TNF-α antibodies, varying doses of EGCG (0-25 µg/ml), 10 nM reduced glutathione, 10 mM NAC, 100 µg/ml SN50 and SN50/M. Cultures were then harvested after 24 hours and cells were lysed in 0.5 ml of TRIZOL Reagent (Invitrogen Inc. Carlsbad, CA, USA) for RNA extraction or in protein lysate buffer for other studies. The cell-free culture supernatants were kept at –70°C.

Treatment with EGCG monocytes viability assay:

The effect of EGCG (0-25 µg/ml) on the viability of monocytes from patients with SLE, TB and SLE-TB was assessed by using MTT Cell Viability Assay Kit (R & D Systems) according to the manufacture's instructions provided.

Reagents supplied in the kit:

Component	Quantity	Storage conditions
MTT reagent	25 ml	2 – 8°C
Detergent reagent	250 ml	18 – 24°C

Assay procedure:

Adherent monocytes were gently scraped with RPMI-1640 medium. After this, monocytes (3 x 10^4 cells/well in 100 µl) were added in 96-well tissue culture plates. Cells were incubated in RPMI-1640 with 2% autologous serum containing varying doses of EGCG for 24 hours at 37°C, 5% CO_2. After 24 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent was added to all wells, including control wells and incubated for two hours in the dark at 20°C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 570 nm using a microplate reader. Control cells were treated exactly the same except that no EGCG added to the wells. The percentage of viable cells was calculated by the formula as described by Islam et al. (2000) and the results are expressed as "Viable cells (% of control cells)".

$$\text{Viable monocytes (\% of control cells)} = \frac{\text{Absorbance value of control cells}}{\text{Absorbance value of treated cells}} \times 100$$

Trypan blue exclusion assay for monocytes viability:

Adherent monocytes were gently scraped with RPMI-1640 medium. Trypan blue suspension (1.6 mg/ml in saline solution) was added to the monocytes at a final concentration of 0.8 mg/ml. The cells were kept at 37°C for 7 minutes in a CO_2 chamber (5%), mounted on a hemocytometer and then observed under light

microscope. The cells taking up Trypan blue (dead cells), and cells excluding the dye (viable cells) were counted. Percentage of viable cells was calculated by the following formula:

$$\% \text{ Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100$$

TNF-α Immunoassay

The concentration of TNF-α in various culture supernatants as well as in serum of patients was determined by use of a commercial ELISA Kit (R & D Systems). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α was available pre-coated onto a microplate. Standards and samples were pipetted into the wells and the immobilized antibody bound any soluble TNF-α present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF- α was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of TNF-α bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

Reagents supplied in the kit:

TNF-α microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against TNF-α.

TNF-α conjugate - 21 ml of polyclonal antibody against TNF-α conjugated to horseradish peroxidase, with preservatives.

TNF-α standard - 10 ng of recombinant human TNF-α in a buffered protein base with preservatives, lyophilized.

Assay diluent RD1F - 6 ml of a buffered protein base with preservatives. It contained a precipitate and was mixed well before and during use.

Calibrator diluent RD6-35 - 21 ml of animal serum with preservatives.

Wash buffer concentrate - 21 ml of a 25-fold concentrated solution of buffered surfactant with preservatives.

Colour reagent A - 12.5 ml of stabilized hydrogen peroxide.

Colour reagent B - 12.5 ml of stabilized chromogen (tetramethylbenzidine).

Stop solution - 6 ml of 2 N sulphuric acid.

Plate covers - 4 adhesive strips.

Working reagents:

Wash buffer - 20 ml of wash buffer concentrate was diluted into deionized or distilled water to prepare 500 ml of wash buffer.

Diluted calibrator diluent RD6-35 - 20 ml of calibrator diluent RD6-35 was mixed with 80 ml of deionized or distilled water to yield 100 ml of diluted calibrator diluent RD6-35.

Substrate solution - Colour reagents A and B were mixed together in equal volumes within 15 minutes of use to form substrate solution. It was protected from light.

TNF-α standard - TNF-α standard was reconstituted with 1.0 ml of distilled water. This reconstitution produced a stock solution of 10,000 pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Assay procedure:

50 µl of assay diluent RD1F was added to each well of 96 well polystyrene microplate coated with a mouse monoclonal antibody against TNF-α. Thereafter, 200 µl of standards, samples, or control per well was added, covered with the adhesive strip provided and incubated for 2 hours at room temperature. The plate was washed four times by filling each well with wash buffer using a squirt bottle.

After washing, 200 µl of TNF-α conjugate was added to each well, covered with a new adhesive strip and incubated for 1 hour for cell culture supernatants and 2 hours for serum samples at room temperature. After four washings with wash buffer, 200 µl of substrate solution was added to each well and incubated for 20 minutes at room temperature in the dark, a blue colour appeared. Thereafter, 50 µl of stop solution was added to each well to stop the reaction. Then the absorbance of each well was determined within 30 minutes, using a microplate reader set to 450 nm. The cut off or lower limit of sensitivity was 4.4 pg/ml.

Glutathione peroxidase assay

The activity of glutathione peroxidase (GPx) was measured as described elsewhere (Mohandas et al., 1984; Mates et al., 1999). The oxidized glutathione (GSSG) produced during GPx reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was regarded as the rate of GSSG formation during the GPx reaction. Patients as well as healthy control monocytes were co-cultured for 24 hours with or without 10 mM NAC, 100 µg/ml SN50, 100 µg/ml SN50/M and 0-25 ug/ml EGCG. Thereafter, cells were gently scraped with lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 500 mM PMSF). The cell suspension was homogenized with a sonicator on ice and centrifuged at 10,000 rpm for 10 minutes. Protein concentrations of supernatants were determined by the method of Bradford with BSA as the standard, and were subjected to GPx activity determination. The reaction mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 unit/ml glutathione reductase, 1.5 mM cumene hydroperoxide, and 20–100 µl of samples were incubated at 25°C for 5 minutes. The reaction was initiated by the addition of cumene hydroperoxide. The kinetic change was spectrophotometrically recorded at 340 nm (37°C) for 3 minutes. GPx activity was calculated after

subtraction of the blank value, as µmol of NADPH oxidized/minute/mg protein (U/mg protein).

Intramonocyte Glutathione Assay.

Glutathione (GSH) levels in treated or control monocytes were assayed by spectrophotometry, using a GSH assay kit (Calbiochem) as previously described by us (Hasan et al., 2007). Monocytes were mixed with equal volume of ice cold 5% metaphosphoric acid and centrifuged at 3000 rpm for 15 min. Supernatants were used for GSH assay, as per the manufacturer's instruction.

Assay for Malondialdehyde (MDA) levels.

As a matter of fact, free radicals, because of their unstable and transient nature are difficult to measure directly. Their tendency to cause lipid peroxidation has been used as an indirect measure. Hence estimation of lipid peroxides (markers of lipid peroxidation), was carried out by measuring MDA (Malondialdehyde) as described earlier (Philpot, 1963).

Principle: One Molecule of MDA reacts stoichiometrically with two molecules of thiobarbituric acid (TBA) at pH 3.5. The pink color chromogen can be measured spectrophotometrically at 532 nm.

Procedure: For assay 1 ml of serum was mixed with 2.5 ml of 20% trichloroacetic acid (TCA) and 1 ml of 0.67% of aqueous solution of TBA. The mixture was heated for30 minutes in boiling water bath, the pink pigment was extracted with 2 ml of n-Butanol and its absorbance was read at 532 nm against n-butonol as blank:

$$MDA \frac{ODofsample}{ODofs\tan dard} x \text{ Conc. of standard MDA}$$

RNA extraction

After lysis of monocytes in 0.5 ml TRIZOL Reagent, the cell lysates were agitated with glass beads to complete cell wall disruption. After cooling on ice the tubes were again subjected to repeated disruption as above. The tubes were cooled and 200 µl of chloroform was added to each sample, followed by vortexing for 2 minutes, and centrifugation at 3000 rpm for 5 minutes. Samples were then transferred to fresh eppendorf tubes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The aqueous layer was harvested and transferred to a fresh tube. After addition of 100 µl Cleanascite (CPG Inc., Lincoln Park, NJ, USA), samples were gently rocked for 10 minutes and then centrifuged at 14,000 rpm for one minute. The aqueous layer obtained was mixed with 500 µl of chloroform-isoamyl alcohol (24:1) and vortexed. RNA was precipitated using 50 µl of 1 M sodium acetate, and 475 µl of isopropanol at –20°C for 3 hours in the presence of glycogen. This was followed by centrifugation at 14,000 rpm and the pellet obtained was washed two times with 75% ethanol, and resuspended in 87 µl DEPC-water. DNAase 1 digestion (10 µl of 10X DNAse 1 buffer in 0.5 M Tris pH 7.5, 0.1 M $MgCl_2$, 1 mM DTT; and 50 µg/ml BSA, 2.0 µl RNAase inhibitor; 10U RNAase free DNAase 1) was employed to remove DNA. The reaction was stopped by the addition of 700 µl of 0.5 M NH_4OAc and the RNA was re-extracted using 500 µl of acid phenol-chloroform (1:1). The aqueous layer was harvested, extracted again with chloroform-isoamyl alcohol and precipitated.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The DNAase-treated RNA was subjected to reverse transcription using oligo(dT) primers with SuperScript II reverse transcriptase (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. RNA (2 µg) was transcribed into cDNA in a 20 µl reaction volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 25

μg/ml oligo$(dT)_{12-18}$ primers and 10 U/μl of SuperScript II reverse transcriptase, at 42°C for 50 minutes. The reaction was then stopped by heating at 70°C for 15 minutes followed by rapid chilling on ice.

PCR: The primers used as described by our laboratory previously (Hasan et al., 2006). cDNA for β-actin was amplified with various primer sets supplied by (Stratagene, La Jolla, CA, USA). For PCR, 2.0 μl of each cDNA sample was used as template in the PCR amplification. The reactions were carried out in a 50 μl reaction volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 2U of Taq DNA polymerase (Invitrogen) and 0.2 μM of each forward and reverse primers. After initial denaturation for 2 minutes at 95°C, 35 cycles at 95°C for 15 seconds, 60°C for 45 seconds were performed, followed by 72°C for 1 minute. The reaction products were visualized by electrophoresis in 2% agarose after staining with 0.5 μg/ml ethidium bromide.

Quantitative real-time RT-PCR:

Real-time quantitative reverse transcriptase PCR (RT-PCR), which is the latest innovation in the field of PCR technology, provides a sensitive, reproducible, and accurate method for determining mRNA levels in tissues or cells. The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing (Heid et al., 1996).

Two important findings led to the discovery of real-time PCR. First, the Taq polymerase has a 5′–3′ exonuclease activity (Holland et al., 1991), apart from its polymerase activity. Second, dual-labelled fluorogenic oligonucleotide probes have been created which emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer (Cardullo et al., 1988). In the TaqMan assay (Applied Biosystems, Foster City, CA, USA), these two principles are combined. In this system a probe, the so-called TaqMan probe, is designed to anneal to the target sequence between the classical forward and

reverse primers. The probe is dually labelled, with a reporter fluorochrome (eg., 5-carbofluorescein, or FAM) at one end and a quencher dye (eg., N,N,N',N'-tetramethyl-6-carborhodamine, or TAMRA) at the other end. In the intact probe, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe will be degraded during the extension phase by the 5'–3' exonuclease activity of the Taq polymerase, separating the reporter and quencher, thus resulting in an increase in reporter fluorescence emission. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

Procedure:

Internal fluorescent hybridization probes were used in ABI Prism 7700 Detection System (ABI/PerkinElmer (PE) Biosystems, Foster City, CA, USA), which allows the sensitive and specific quantification of individual host (Hartel et al., 1999), as well as *M. tuberculosis* RNA, transcripts (Wilkinson et al., 2001) by quantitative real-time RT-PCR. TaqMan™ PCR primers and probes as well as target-specific RT primer for each assay were designed as described elsewhere (Wilkinson et al., 2001; Islam et al., 2004). We have reported the primer and probe sequences used for R18, TNF-alpha and iNOS earlier (Islam et al., 2004, Hasan et. al., 2006; Singh et al., 2002), and are also given below. All probes were dually labeled with FAM at the 5' end and TAMRA at the 3' end. The proximity of the dye (FAM) and the quencher (TAMRA) on the intact probe prevents detection of any fluorescence. However, degradation of the probe during the course of PCR allows the release and detection of FAM (Holland et al., 1991). The primer and probe sequences used for R18, TNF-alpha and iNOS were as given below:

R18:

RT primer: GACGGTATCTGATC;
reverse primer: 5′-CAT TCT TGG CAA ATG CTT TC-3′;
forward primer: 5′-CGC CGC TAG AGG TGA AAT TC-3′;
TaqMan probe: 5′-6FAM-ACC GGC GCA AGA CGG ACC AGA-TAMRA-3′.

TNF-alpha:

RT primer: GGTTTCTACAACA;
reverse primer: 5′-GTTCGAGAAGATGATCTGACTGCC-3′;
forward primer: 5′-AGGCGGTGCTTGTTCCTCA-3′;
TaqMan probe: 5′-6FAM-CCAGAGGGAAGAGTTCCCCAGGGAC-TAMRA-3′.

iNOS:

RT primer: 5′-CTCTggTCAAAC-3′;
forward primer: 5′-AgCggg-ATgACTTTCCAAgA-3′;
reverse primer: 5′-ATAATggACCCCAgg-CAAgATT-3′
TaqMan Probe: 5′-AMCCATAAggCCAAAgggATTTTAACTTgCAg-3′

The PCRs for all amplifications were similar: 5 µl of each cDNA, 20 µl of Taqman Universal PCR Master Mix (PE Biosystems), which contains optimal amounts of AmpliTaq Gold DNA polymerase (which protects against amplicon carryover) and of dNTPs, and optimal amounts of probe and primers calibrated to allow measurement of the targets. First, cDNA was synthesized in the presence of 0.5 µl of murine leukemia virus enzyme (Invitrogen, USA)/reaction and 10 µM each RT primer, dNTPs, and other substrate. Conditions for PCR were similar for all products (1 cycle of 2 minutes at 50°C and 1 cycle of 10 minutes at 95°C and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C). The cycle threshold for each sample was compared with the cycle threshold values of known amounts of a standard DNA constructed for each target and amplified simultaneously. To assure lack of DNA contamination in the RNA samples, in some experiments, a duplicate tube of sample with no RT enzyme was included as control. DNA contamination remained negligible. In each sample, host 18S ribosomal RNA was used as the internal control. Expression of TNF-α mRNA and iNOS were corrected to internal control (host 18S rRNA) in the same sample and was expressed as copies of TNF-α or iNOS in 10^{10} copies of R18 (equivalent to 1 x 10^6 monocytes).

Statistical analysis:

Results were analyzed by paired t-test and the data expressed as mean ± SEM of six to eight experiments unless otherwise specified. $P<0.05$ was considered statistically significant.

RESULTS

RESULTS

The present study was carried out in four distinct parts – (I) evaluation of clinical profile of patients whose blood or serum were undertaken in this study was carried out, (II) characterization of monocytes from patients with SLE, TB and SLE-TB whose untreated / treated monocytes in turn would be utilized as valuable immuno-reagents, (III) regulation / modulation of above monocytes and (IV) immunological investigations of the above characterized / modulated monocytes and clinically evaluated sera's.

Thus, first of all, prior to any study, evaluation of clinical profile of patients whose blood or serum were undertaken in this study was carried out, which are described as under:

(I): CLINICAL PROFILE OF PATIENTS

A. Classification of patients

A total of 100 subjects were selected for clinical evaluation, where 10 were healthy normal subjects that served as controls. The remaining ninety study group patients (100%) comprised of 30 SLE patients (33.3%), 30 TB patients (33.3) and 30 SLE-TB patients (33.3%).

B. Distribution of sex in clinically classified patients

Next, the patients and control subjects undertaken in this study were distributed with respect to sex. Thus, out of 10 healthy normal subjects, 5 were males (50%) and 5 females (50%). The 30 SLE patients comprised of 10 males (33.3%) and 20 females (66.6%). Similarly, out of 30 TB patients, 10 were males (33.3%) and 20 females (66.6%). The same is true for SLE-TB patients, where out of 30 such patients, 10 were males (33.3%) and 20 were females (66.6%).

C. Distribution of Age

The above clinically classified patients were of 4 age groups, and are summarized in Table 3. Briefly, the 4 types of age groups were: Group 1= 20-35

yrs, Group 2 = 36-50 yrs, Group 3 = 51-65 yrs and Group 4 = 66-80 yrs. In control subjects, 20% subjects were from age group 1, whereas 30% each were from age group 2 and 3 respectively. Finally, there were 20% subjects from age group 4.

D. Montoux Test

The initial detection of TB infection was detected by Montoux test. The following Table IV shows the distribution of Montoux Test (+ve/-ve) in control, TB and SLE-TB patients. None of the healthy control subjects or SLE patients responded towards Montoux test. On the contrary, 100% TB patients as well as SLE-TB selected in this study showed a positive Montoux test. The observation of positive Montoux test in TB and SLE-TB patients selected in this study indicates the presence of bacilli infection in the selected host. The distribution of Montoux test is depicted in Table 1.

TABLE I

Distribution of Montoux Test in control, TB, SLE and SLE-TB patients

Groups	No. of cases	Montoux Test	
		Positive	Negative
Normal healthy control	10	Nil	10 (100%)
SLE	30	Nil	30 (100%)
TB	30	30 (100%)	Nil
SLE-TB	30	30 (100%)	Nil

E. Blood Glucose levels in SLE, TB and SLE-TB patients

All the patients and control samples undertaken in this study were subjected to evaluation of blood glucose levels. Healthy normal subjects showed normal blood glucose levels. Interestingly, 16% SLE patients of age group 51-65 yrs (both sex) exhibited an slightly augmented blood glucose level (mean range: 167-183 mg%). Similarly, 19% TB patients of age group 66-80 yrs showed to have elevated blood glucose (mean range: 171-187 mg%). Also, 20% patients having both SLE along with TB (SLE-TB) in the age group of 51-65 yrs and 66-80 yrs showed an enhanced blood glucose level falling under the mean range of 173-193 mg/%.

F. Distribution of Anti-dsDNA

Direct binding ELISA analyzed patients and control samples undertaken in this study for anti-dsDNA evaluation. The observations made are summarized in Table VI. Healthy normal subjects showed negative anti-dsDNA results as the titre of anti-dsDNA was found to be less that 1:100. It is to be pointed out here that any anti-dsDNA titre, which is equal to or greater than 1:1600, is considered to be positive. Similarly, all the TB patients selected in this study exhibited an anti-dsDNA titer of <1:800, thereby suggesting for the fact that none of the TB patient's sera had any anti-dsDNA antibody in it. On the contrary, both SLE and SLE-TB patient's sera showed an anti-dsDNA titre that was greater than 1:128000, thereby indicating for the presence of high titer of anti-DNA autoantibodies ($P<0.001$).

G. Chest X-Ray

SLE patients had no evidence of cavity formation/fluffy shadow on chest X-ray to suggest pulmonary kochs. On the contrary, both TB and TB-SLE groups of patients exhibited pulmonary kochs. The summary is depicted below in Table II

TABLE – II

Distribution of Chest X-ray in TB, SLE-TB and SLE patients

Groups	Cases		Chest X-ray
	No	%Age	Mean observation
Normal Control	10	100	No cavity / fluffy shadow
TB	30	33.3	Cavity/fluffy shadow in all cases
TB-SLE	30	33.3	Cavity/fluffy shadow in all cases
SLE	30	33.3	No cavity/fluffy shadow

(II) CHARACTERIZATION OF MONOCYTES FROM NORMAL HEALTHY AND PATIENTS WITH SLE, TB AND SLE-TB:

1. Comparative study on the human housekeeping gene of human monocytes from normal controls versus patient with SLE, TB and SLE-TB:
Prior to any investigation, an attempt was made to probe the adverse effect of disease activity in monocytes of patients with SLE (n=3; $P<0.001$), TB (n=3; $P<0.001$) and SLE-TB (n=3; $P<0.001$) in comparison to normal healthy controls (n=3), if any, on the human housekeeping gene. It was observed that disease activity in 24 hr monocyte cultures failed to exert any adverse effect on the host housekeeping genes like R18 gene (18S rRNA) as revealed by quantitative real-time RT-PCR (Fig. 1).

2. Basal levels of TNF-α expressions at the gene and protein levels in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB.

Thereafter, we investigated the basal levels of TNF-α mRNA expressions in 24 hr monocyte cultures from normal healthy controls (n=8) and patients with SLE (n=8) TB (n=8) and SLE-TB (n=8). TNF-α mRNA was corrected to host internal control, i.e., 18S rRNA, in the same sample. As evident from Fig. 2, the basal levels of TNF-α mRNA copy numbers in patients with SLE, TB and SLE-TB were recorded as 6.6 logs ($P<0.001$), 6.3 logs ($P<0.001$) and 7.7 logs ($P<0.001$) respectively. No or negligible TNF-α mRNA expression was observed in normal healthy controls (Fig. 2).
Thus, the magnitude of TNF-α mRNA expression in monocytes was of the order of SLE-TB > SLE > TB patients respectively.

Next, we further probed the basal secreted TNF-α expressions in culture supernatants of the above monocyte cultures that were harvested after 24 hrs. As evident from Fig. 3, the concentration of soluble TNF-α secreted in supernatants was observed as 194.3 ($P<0.001$), 183.2 ($P<0.001$) and 221.2 ($P<0.001$) pg/ml from patients with SLE (n=8), TB (n=8) and SLE-TB (n=8) respectively. Again, no or negligible TNF-α protein expression was observed in normal healthy controls (n=8) (Fig. 3).

3. Basal levels of iNOS mRNA expressions in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB.

Next, we investigated the basal levels of iNOS mRNA expressions in 24 hr monocyte cultures from normal healthy controls (n=8) and patients with SLE (n=8) TB (n=8) and SLE-TB (n=8). iNOS mRNA was corrected to host internal control, i.e., 18S rRNA, in the same sample. As evident from Fig. 4, the basal levels of iNOS mRNA copy numbers in patients with SLE, TB and SLE-TB were recorded as 5.3 logs ($P<0.001$), 5.4 logs ($P<0.001$) and 5.9 logs ($P<0.001$) respectively. No or negligible iNOS mRNA expression was observed in normal healthy controls (Fig. 4). Thus, the magnitude of iNOS mRNA expression in monocytes was of the order of SLE-TB > SLE > TB patients respectively.

4. Basal levels secreted IFN-γ protein expressions in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB.

Next, we further probed the basal secreted IFN-γ expressions in culture supernatants of the above monocyte cultures that were harvested after 24 hrs. In comparison to normal healthy control cultures (167.85 pg/ml), the concentration of soluble IFN-γ was found to be down-regulated to 43.56 ($P<0.001$), 41.23 ($P<0.001$) and 38.26 ($P<0.001$) pg/ml in cultures from patients with SLE (n=8), TB (n=8) and SLE-TB (n=8) respectively (Fig. 5).

5. Glutathione peroxidase (GPx) activity in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB.

Next, an attempt was made to probe the glutathione peroxidase (GPx) activities in culture supernatants of the above monocyte cultures that were harvested after 24 hrs. Normal healthy control cultures exhibited a GPx activity of 57.22 U/mg proteins (Fig. 6). As expected, GPx activities were suppressed to the order of 29.25 ($P<0.001$), 25.14 ($P<0.001$) and 20.48 ($P<0.001$) U/mg protein in cultures from patients with SLE (n=8), TB (n=8) and SLE-TB (n=8) respectively (Fig. 6).

(III) REGULATION STUDY:

1. Dose response effect of NAC on the expressions of secreted TNF-α protein in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB.

Monocytes obtained from patients with SLE (n=3), TB (n=3) and SLE-TB (n=3) were co-cultured in RPMI-1640 media with varying doses of NAC (0-10 mM) for

24 hrs at 37C/5%CO_2. Thereafter, the cells were harvested and subjected to secreted TNF-α protein expression analysis by ELISA. As expected, normal healthy control cells failed to show any effect of NAC as no or negligible TNF-α was recorded in untreated cells. However, NAC exhibited an appreciable dose-dependent suppression in secreted TNF-α protein expressions in 24 hr monocyte cultures from patients with SLE (n=3), TB (n=3) and SLE-TB (n=3) respectively (Fig. 7; $P<0.001$ for all three groups).

2. Toxicity assessment of EGCG:

Prior to probing the effect of EGCG, an attempt was made to check the toxic effect, if any, monocytes from patients with SLE (n=3), TB (n=3) and SLE-TB (n=3) respectively. For this purpose, we chose a dose in between 0 – 25 μg/ml of EGCG (dose-response assay) as this dose has previously been established in our laboratory in healthy monocytes. The results depicted in Fig. 8 ($P<0.001$), failed to show any toxic effect of EGCG (0 – 25 μg/ml) on monocytes as revealed by MTT (Fig. 8) or trypan blue exclusion assays. Cell viability is expressed as percent (mean ± SEM) viable cells compared to untreated cells (taken as 100% viable). Also, no effect was observed on housekeeping genes like R18 (18S rRNA) by quantitative real-time RT-PCR (Fig. 9), thereby indicating that EGCG did not non-specifically affect human TNF-α transcription.

3. Dose response effect of EGCG on the expressions of secreted TNF-α protein in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB.

Next, monocytes from patients with SLE (n=5), TB (n=5) and SLE-TB (n=5) were co-cultured with varying doses of EGCG (0-25 μg/ml) for 24 hrs at 37C/5%CO_2. After harvesting, the cells were subjected to analysis of secreted TNF-α protein expression by ELISA.

As expected, normal healthy control cells failed to show any effect of EGCG as no or negligible TNF-α was recorded in untreated cells. However, EGCG exhibited an appreciable dose-dependent suppression in TNF-α expressions in 24 hr monocyte cultures from patients with SLE, TB and SLE-TB respectively (Fig. 10; P<0.001 for all). The IC_{50} was computed out to be ~ < 5 μg/ml.

4. Effect of SN50 and SN50M on the expressions of secreted TNF-α in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB in order to probe the role of NF-κB:

Induction of TNF-α as a consequence of cellular activation is mediated via NF-κB (Toossi et al., 1997; Fan et al., 2002, Hasan et al., 2006). Reports indicate that TNF-α induced nuclear translocation of NF-κB was inhibited in SN50 peptide-treated human monocytic cell lines as demonstrated in EMSA (Lin et al., 1995). In view of it, we employed SN50, an inhibitor of NF-κB, to assess the role of NF-κB in activation of TNF-α protein expression in *M. tuberculosis*-infected monocytes. SN50 (100 μg/ml) was added to monocytes 3 minutes prior to *M. tuberculosis* infection. Control cultures did not receive SN50. At 24 hours, SN50 suppressed secreted TNF-α protein expression by around 1.96-folds (i. e. suppressed from 194.3 pg/ml to 98.9 pg/ml), 2.11-folds (i. e. suppressed from 183.2 pg/ml to 86.8 pg/ml) and 2.04-folds (i. e. suppressed from 212.2 pg/ml to 103.6 pg/ml) in 24 hr monocyte cultures from patients with SLE (n=5; P<0.001), TB (n=5; P<0.001) and SLE-TB (n=5; P<0.001) respectively (Fig. 11).

Furthermore, in order to ensure that cellular inhibition was not non-specific, we compared the effect of SN50 with its inactive analogue, SN50/M at the same concentration. SN50/M did not affect secreted TNF-α protein expression (P<0.001) (Fig. 11). Therefore, it can be inferred that the increased expression of TNF-α in 24 hr monocyte cultures from patients with SLE, TB and SLE-TB respectively is mediated mainly via NF-κB.

5. Effect of NAC and EGCG on GPx activity in 24 hr monocyte cultures from normal healthy controls and patients with SLE, TB and SLE-TB.

Next, an attempt was made to probe the modulatory effect of NAC and EGCG on glutathione peroxidase (GPx) activities in culture supernatants of the above monocyte cultures that were harvested after 24 hrs. As described above in Fig. 6, normal healthy control cultures exhibited a GPx activity of 57.22 U/mg proteins, whereas GPx activities were suppressed to the order of 29.25, 25.14 and 20.48 U/mg proteins in cultures from patients with SLE (n=8), TB (n=8) and SLE-TB (n=8) respectively (Fig. 6). On the contrary, modulation with 10 mM NAC and 5 µg/ml of EGCG showed interesting results. Thus, as evident from Fig. 12, NAC (10 mM) showed amelioration in GPx activities from 29.25, 25.14 and 20.48 U/mg proteins to 44.23, 42.01 and 40.88 U/mg proteins in cultures from patients with SLE, TB and SLE-TB respectively (Fig. 12). Similarly, EGCG (5 µg/ml) exhibited showed amelioration in GPx activities from 29.25, 25.14 and 20.48 U/mg proteins to 49.23, 46.01 and 45.88 U/mg proteins in cultures from patients with SLE, TB and SLE-TB respectively (Fig. 13) ($P<0.001$ for all).

6. Determination of MDA levels in 24 hr culture filtrate of monocytes of patients with SLE, TB and SLE-TB

Thereafter, an attempt was also made to evaluate the MDA levels in culture supernatants of 24 hr monocyte cultures from patients with SLE (n=3), TB (n=3) and SLE-TB (n=3) respectively. As shown in Fig. 14, normal healthy control (n=3) cultures exhibited an MDA level to the order of 8.12 ng/ml (Fig. 14). On the contrary, augmented MDA levels in 24 hr monocyte cultures supernatants from patients with SLE (n=3), TB (n=3) and SLE-TB (n=3) were found, which were of the order 18.22 ng/ml (2.24-folds; $P<0.001$), 17.34 ng/ml (2.12-folds; $P<0.001$) and 22.26 ng/ml (2.74-folds; $P<0.001$) respectively (Fig. 14).

7. NAC and EGCG-induced modulation of MDA levels in 24 hr culture filtrate of monocytes of patients with SLE, TB and SLE-TB

To have further in-sight, an attempt was also made to probe the modulatory effect of NAC and EGCG on augmented levels of MDA in culture supernatants of the above monocyte cultures that were harvested after 24 hrs. As described above in Fig. 14, normal healthy control cultures exhibited 8.12 ng/ml of MDA, and augmented to the order of 18.22 ng/ml, 17.34 ng/ml and 22.26 ng/ml in cultures from patients with SLE (n=3), TB (n=3) and SLE-TB (n=3) respectively. On the contrary, modulation with 10 mM NAC showed down-regulation in MDA levels to 10.32 ng/ml. 9.34 ng/ml and 9.01 ng/ml in cultures from patients with SLE, TB and SLE-TB respectively (Fig. 15). Similarly, EGCG (5 ug/ml) exhibited down-regulation in MDA levels to 8.23 ng/ml. 7.89 ng/ml and 7.12 ng/ml in cultures from patients with SLE, TB and SLE-TB respectively (Fig. 16, $P<0.001$ for all three groups of patients).

(IV) IMMUNONOLICAL STUDY:

1. Immuno-binding of dsDNA antigens by antibodies present in sera of patients with SLE by direct binding and inhibition-ELISA:

The binding of naturally occurring SLE autoantibodies with a variety of antigens, ranging from native calf thymus purified DNA (free of proteins and single stranded regions) to mycobacterial protein antigens was probed by direct binding and inhibition ELISA. Prior to any study, SLE sera (n = 40) were screened for their activity. Only those SLE sera exhibiting a titer of greater than 1:6400 (n=20) against native DNA (Fig. 17) were selected for determining the reactivity against mycobacterial protein antigens. It is to be pointed out that apart from showing high magnitude specificity towards native DNA, the selected SLE sera having anti-DNA antibody also showed appreciable degree of reactivity towards a wide spectrum of antigens. Also, it's worth mentioning out here that prior to any ELISA experiments, it was ensured that the secreted mycobaterial proteins were free

from any contaminants of DNA due to possible leakage from ruptured bacilli, which in turn, may yield false positive test. Colorimetric estimations showed absence of any DNA contaminations in the culture filtrate having secreted proteins (data not shown). Thereafter, direct binding ELISA on microtiter plates coated with intracellular and secreted mycobacterial protein antigens exhibited an anti-DNA antibody titer of greater that 1:12800 (Fig. 18, 19; $P<0.001$ for all). Normal human sera, which acted as corresponding controls failed to show any appreciable reactivity. Surprisingly, the binding curves showed augmented reactivity of SLE anti-DNA antibodies against mycobacterial protein antigens than against native dsDNA.

Next, after observing high binding with SLE sera, an attempt was also made to check the binding with Protein-A-Sepharose purified SLE IgG. The binding curves by direct binding ELISA on plates coated with secreted mycobacterial protein antigens against SLE IgG (n=4) were of similar pattern observed with SLE sera (Fig. 20; $P<0.001$).

After determining the reactivity of SLE anti-DNA antibodies by direct binding ELISA, an attempt was also made to determine the specificity of the above autoantibodies in SLE sera by employing competition-inhibition ELISA. A variety of inhibitors ranging from nucleic acids to mycobacterial proteins were employed in inhibition ELISA. As evident from Fig. 21, double stranded DNA showed a maximum inhibition in the anti-DNA antibody binding to the order of 73.11 percent ($P<0.001$) at a maximum inhibitor concentration of 10 µg/ml. Here, fifty percent inhibition in the antibody activity was observed at 2.1 ug/ml. Similar maximum inhibition in antibody activity was observed with heat denatured single stranded DNA (71.33%; $P<0.001$), but fifty percent inhibition in antibody activity was recorded a much lower ssDNA inhibitor concentration (1.2 ug/ml). Next, no appreciable inhibition in antibody activity was observed when RNA was employed as an inhibitor (Fig. 22; $P\leq05$).

Thereafter, secreted mycobacterial protein antigens(s), were employed as inhibitors. As evident from Fig. 23, 82.13 percent inhibition in SLE IgG activities respectively were recorded at maximum inhibitor concentrations of 10 µg/ml ($P<0.001$). Fifty percent inhibition in SLE IgG activities was recorded at an inhibitor concentration of 0.01 µg/ml. Next, the above observations were further substantiated from results obtained by employing immunoaffinity purified (DNA-polylysyl-sepharose 4B column) anti-DNA antibody (SLE IgG) in the binding studies (Fig. 24). It may be pointed out here that the lower the inhibitor concentration at which fifty percent inhibition is achieved, the greater the specificity of the concerned antibody towards the concerned antigen/inhibitor. Thus, judged on the basis of fifty percent inhibition, the binding curves indicate the presence of highly specific autoantibodies in SLE sera for *Mycobacterium tuberculosis* $H_{37}Rv$ secreted protein antigen (s). Thus, the above results show that the binding of SLE anti-DNA autoantibodies were of greater magnitude towards mycobacterial secreted protein antigens as compared to native DNA.

Next, in sharp contrast to the above, when anti-TB antibodies (sera from TB patients, n=30) were employed on plates coated with dsDNA, then no binding was observed (Fig. 25) in direct binding ELISA. Since, DNA binding with TB sera was not observed in direct binding ELISA, thus, inhibition ELISA was not carried out.

2. Immuno-binding of MTB 30kDa antigens by antibodies present in sera of patients with SLE and TB by inhibition-ELISA:

All the TB sera's (n=30) undertaken in this study were subjected to specificity determination against respective antigens and inhibitors i. e. tuberculosis 85B antigen (30kDa) for TB sera and dsDNA for SLE patients respectively, by employing competition-inhibition ELISA.

Inhibition ELISA on plates coated with mycobacterium tuberculosis 85B antigen (30kDa) against sera of patients (n=30) with TB indicated interesting results

Nearly all the TB sera employed in this study exhibited a maximum of 84 percent inhibition ($P<0.001$) in anti-TB antibody activity at a maximum inhibitor (i.e. MTB 30kDa) concentration of 10 µg/ml (Fig. 26). Fifty percent inhibition here was achieved at an inhibitor concentration of 0.1 µg/ml, thereby indicating the high specificity of TB antibodies in TB sera against MTB 30kDa.

Next, after selecting high specificity sera's of TB and SLE patients as described above, an attempt was made to evaluate the correlation, if any, between TB and autoimmune SLE. For this purpose, we investigated the (a) reactivity/specificity of anti-DNA antibodies found in SLE sera (n=20) against mycobacterial Ag85B (30 kDa) in comparison to dsDNA, (b) reactivity/specificity of anti-TB antibodies found in TB sera (n=20) against dsDNA in comparison mycobacterial Ag85B (30 kDa), and (c) comparative reactivity of dsDNA versus MTB 30 kDa against sera from patients having both TB and SLE (n=20).

As evident from Fig. 27, sera of patients with SLE (n=30) exhibited a maximum of 87.7% ($P<0.001$) inhibition with dsDNA on microtiter ELISA plates coated with dsDNA, where 50% inhibition in anti-DNA antibody activity was recorded at an inhibitor concentration of 0.08 ug/ml (Fig. 27). Interestingly, when MTB 30kDa was employed as an inhibitor on plates coated with dsDNA, then a magnitude of binding was observed with anti-DNA Abs found in SLE sera. A maximum of 78.2% inhibition ($P<0.001$) in anti-DNA activity was recorded by 10 ug/ml of mycobacterial 30kDa antigen/inhibitor, where 50% inhibition was achieved at an inhibitor concentration of 0.09 µg/ml (Fig. 27). On the contrary, anti-TB antibodies found in TB sera (n=30) showed low magnitude recognition with dsDNA on plates coated with MTB 30kDa. Here only 29% ($P>0.05$) inhibition in anti-TB antibodies was recorded with dsDNA (Fig. 28), and that, 50% inhibition could not be achieved. However the same anti-TB antibodies on the same antigen (30 kDa) coated plates showed high magnitude antibody recognition (83%; $P<0.001$) when MTB 30kDa was employed as an inhibitor (Fig. 28). Thus, the results are indicative for an appreciable immunological correlation between SLE and TB. To

have further insight, inhibition-ELISA against dsDNA and MTB 30 kDa was carried out by using antibodies present in sera of patients having both SLE and TB. Here, a maximum of 95% and 93% ($P<0.001$) inhibition was observed with dsDNA and MTB 30kDa inhibitors respectively on plates coated with MTB 30 kDa antigen against Abs found in sera of patients having both the diseases i. e. SLE and TB (TB-SLE) (n=30) (Fig. 29). Fifty percent inhibition in both cases was recorded at very low inhibitor concentrations (0.07 µg/ml for both). The data depicted in Fig 29 are again indicative for the close immunological relationship between SLE and TB.

3. Immuno-recognition of antigens found in human monocytes of SLE and TB patients by anti-dsDNA Abs and anti-TB Abs found in patients with SLE and TB

Next, we also carried out a number of inhibition-ELISA's to probe the binding effects by employing antigen / inhibitors obtained from cell lysates of human monocytes from TB and SLE patients. Figures 30 and 31 illustrates the binding of anti-TB antibodies and anti-DNA Abs found in sera of patients with TB (n=5) and SLE (n=5) respectively with protein lysates obtained from monocytes (0.5 x 10^6/well) from blood of respective TB and SLE patients that were cultured for 24 hrs in 12-well tissue culture plates.

Here, a maximum of 91.8% ($P<0.001$) and 95.9% ($P<0.001$) inhibitions in anti-TB and anti-dsDNA Ab activity respectively were recorded at a maximum inhibitor concentration of 10 ug/ml, where 50% inhibitions in the anti-TB Ab activity was achieved at a low inhibitor concentration of 0.08 ug/ml (Fig. 30), whereas that for anti-DNA from SLE sera was achieved at an inhibitor concentration of 0.07 ug/ml (Fig. 31). A further augmented inhibition (~ 97%; $P<0.001$) in anti-TB antibody activity was observed with sera of patients having both TN and SLE (n=5) (Fig. 32) at a maximum inhibitor concentration of only 1 µg/ml, and that, 50% inhibition

in this case was recorded at a much low inhibitor concentration of 0.01 µg/ml (Fig. 32).

4. Reduced Glutathione-induced immuno-suppression studies

In view of the role of reactive oxygen species (ROS) in both autoimmune SLE and tuberculosis (TB) being well documented, an attempt was made in the present study to neutralize/suppress the ROS induced effects in monocytes of patients with TB and SLE by employing reduced glutathione (r GSH), which is a known *in vivo* antioxidant. Thus, for this purpose, monocytes from blood of patients with SLE (n=5), TB (n=5) and SLE-TB (n=5) were isolated as described in methods and were adhered onto 12-well tissue culture plates and cultured with or without 10 nM of reduced glutathione for 24 hrs at 37 $^{\circ}$C / 5%CO_2. Thereafter, treated/untreated monocytes were lysed in 1 ml/well of protein lysis buffer and were subsequently used as antigen / inhibitor in inhibition ELISA against antibodies in sera with SLE or TB or SLE-TB.

As evident from Fig. 33, co-culturing of monocytes obtained from TB patients with 10 nM of reduced glutathione for 24 hrs resulted in the tremendous amount of down-regulation of recognition of anti-TB antibodies by cell lysates of the above cultured monocytes. Here, only a maximum of 32% ($P>0.01$) inhibition in anti-TB antibody activity was recorded at a maximum inhibitor concentration of 20 µg/ml, and that, 50% inhibition could not be achieved here (Fig. 33). Thus, when compared to the data in Fig 30, reduced glutathione suppressed the anti-TB antibody activity (Fig. 33) by around 58%. Similarly, in comparison to binding results depicted in Fig. 31, reduced glutathione down-regulated the anti-DNA antibody activity in monocytes of SLE patients by ~ 53% ($P<0.01$) (Fig. 34). On the other hand, reduced glutathione suppressed the anti-TB antibody activity in monocytes of patients with SLE-TB by ~ 36% ($P<0.01$) (Fig. 35) in comparison to untreated monocytes of TB-SLE patients whose data are depicted in Fig. 32). Thus, the results indicate the high magnitude suppression in respective antibody

binding (i. e. abs in sera of patients with SLE or TB or SLE-TB) to corresponding antigens by reduced glutathione.

5. Binding studies with anti-TNF-α antibody

In view of the fact that ROS activates the production of autocrine proinflammatory cytokine, namely TNF-α, which in turn, is involved in the pathogenesis of TB and SLE, thus an attempt was made in the present study to evaluate the binding of anti-TNF-α antibody with various antigens prepared and used in the above-mentioned modulatory studies. Figures 36-38 demonstrates the binding of anti-TNF-α antibody with protein lysates of monocytes from patients with TB (n=5), SLE (n=5) and TB-SLE (n=5).

A maximum of 68.9% ($P<0.001$) inhibition in anti- TNF-α antibody activity was recorded at a maximum inhibitor (i. e. lysates of monocytes from TB patients) concentration of 1 µg/ml, where 50 % inhibition was achieved at an inhibitor concentration of 0.08 µg/ml ($P<0.001$) (Fig. 36). On the other hand, a maximum of 76.5% ($P<0.001$) inhibition in anti- TNF-α antibody activity was recorded at a maximum inhibitor (i. e. lysates of monocytes from SLE patients) concentration of 10 µg/ml, where 50 % inhibition was achieved at an inhibitor concentration of 0.09 µg/ml ($P<0.001$) (Fig. 37). It is noteworthy to observe that a maximum of 80.09 % ($P<0.001$) inhibition in anti- TNF-α antibody activity was achieved at a maximum inhibitor (i. e. lysates of monocytes from SLE-TB patients) concentration of only 0.1 µg/ml, where 50 % inhibition was recorded at an inhibitor concentration of 0.05 µg/ml ($P<0.001$) (Fig. 38).

Co-culturing of monocytes obtained from patients having TB (n=5), SLE (n=5) and TB-SLE (n=5) with reduced glutathione (10 nM) for 24 hrs resulted in the suppression of recognition of anti-TNF- α Ab. Here, only a maximum of 17.26%, 28.03% and 32.01% ($P>0.01$for all) inhibition in anti- TNF-α antibody activity was recorded as is evident from Figs. 39-41, respectively.

6. Measurement of secreted TNF- α in 24 hr culture supernatants of monocytes treated with reduced glutathione.

Level of secreted TNF- α in culture supernatants was found to be greatest in cultures of monocytes from patients with TB-SLE, followed by cultures of monocytes of SLE and TB patients respectively ($P<0.001$ for all) (Fig. 42). The order of reduced glutathione-induced suppression in secreted TNF- α in monocyte culture supernatants was TB > SLE > TB-SLE ($P<0.001$) (Fig. 42). It is noteworthy to observe that secreted TNF-alpha was highest in culture supernatants of patients belonging to category III (Age: 51 yrs to 65 yrs), followed by category II (Age: 36 yrs to 50 yrs) and category I (Age: 20 yrs to 35 yrs) respectively (Fig. 42).

7. Binding of anti-DNA antibodies against dsDNA antigen/inhibitor isolated from monocytes of SLE patients that were co-cultured for 24 hr with NAC, EGCG, SN50 or SN50M.

DNA was isolated by Qiagen Kit from untreated / treated monocytes from patients with SLE (n=5), and were subsequently employed as coating antigen / inhibitor in inhibition-ELISA. As evident from Fig. 43, the specificity of anti-DNA antibodies against dsDNA isolated from SLE monocytes that were co-cultured for 24 hr with 10 mM NAC was found to decrease markedly to 41.2 percent ($P<0.001$) when compared to control untreated monocytes (73.45 percent) (Fig. 43). Here, fifty percent inhibition could be achieved with any of the inhibitor concentrations employed. Next, dsDNA isolated from SLE monocytes (n=5) that were co-cultured for 24 hr with 5 ug/ml EGCG was employed as coating antigen / inhibitor, then a maximum of only 32.45 percent inhibition ($P<0.001$) was observed (Fig. 44). Again, here fifty percent inhibition could not be achieved. The results thus indicate the positive involvement of reactive oxygen species (ROS) in SLE. which was appreciably neutralized / inhibited by NAC and EGCG.

Thereafter, for reasons as described in modulation studies earlier, an attempt was also made to probe the immuno-binding of anti-DNA antibodies against dsDNA isolated from SLE monocytes (n=5) treated with SN50 and SN50M. As evident from Fig. 45, anti-DNA antibodies exhibited a non-significant binding with SN50 treated SLE monocytes (39.22 percent). On the contrary, treatment with control peptide SN50M exhibited high binding as is evident by 69.44 percent inhibition in anti-dsDNA antibody activity (Fig. 45, $P<0.001$). Thus, the activation of NF-kB and in turn, its involvement in SLE is substantiated by the above immuno-binding data.

8. Anti-DNA antibody binding against dsDNA antigen/inhibitor isolated from monocytes of tuberculosis (TB) patients that were co-cultured for 24 hr with NAC, EGCG, SN50 or SN50M.

As mentioned above, DNA was isolated from untreated / treated monocytes from patients with TB (n=5), and were subsequently employed as coating antigen / inhibitor in inhibition-ELISA. As evident from Fig. 46, the specificity of anti-DNA antibodies against dsDNA isolated from TB monocytes that were co-cultured for 24 hr with 10 mM NAC was found to decrease markedly to 46.56 percent ($P<0.001$) when compared to control untreated monocytes (79.33 percent) (Fig. 46). Fifty percent inhibition could not be achieved with any of the inhibitor concentrations employed. Similarly, dsDNA isolated from monocytes of TB patients (n=5) that were co-cultured for 24 hr with 5 µg/ml EGCG, and in turn, were employed as coating antigen / inhibitor in ELISA. The results show a maximum of only 29.45 percent inhibition in comparison to cells devoid of EGCG (maximum inhibition to the order of 79.33%) ($P<0.001$) (Fig. 47). Again, here fifty percent inhibition could not be achieved. The results thus indicate the positive involvement of reactive oxygen species (ROS) in TB, which was appreciably neutralized / inhibited by NAC and EGCG. The results also indicate involvement of mycobacterial antigen(s) in SLE as is evident from high binding of anti-DNA antibodies with mycobacterial DNA.

Thereafter, as described in modulation studies earlier, an attempt was also made to probe the immuno-binding of anti-DNA antibodies (n=5) against dsDNA isolated from TB monocytes (n=5) treated with SN50 and SN50M. As evident from Fig. 48, anti-DNA antibodies exhibited a maximum inhibition of only 35.39 percent with SN50 treated TB monocytes ($P<0.005$). On the contrary, DNA isolated from monocytes treated with control peptide SN50M exhibited high binding as is evident by 71.42 percent inhibition in anti-dsDNA antibody activity (Fig. 48, $P<0.001$). Thus, the activation of NF-kB and in turn, its involvement in TB is substantiated by the above immuno-binding data. This is in accordance with earlier reports from our laboratory at the molecular level (Hasan et al., 2006).

9. Cross reactivity of anti-TB antibodies against dsDNA antigen/inhibitor isolated from monocytes of SLE patients that were co-cultured respectively for 24 hr with NAC, EGCG, SN50 or SN50M.

Next, after ascertaining the immuno-binding of anti-DNA antibodies from SLE patients, an attempt was made to evaluate the specificity / binding of anti-TB antibodies found in patients with TB against DNA isolated from untreated/treated monocytes of patients with (i) SLE, (ii) TB and (iii) SLE-TB.

Inhibition-ELISA results show no or negligible binding of anti-TB antibodies found in sera of patients with TB (n=5) against dsDNA isolated from untreated / treated monocytes of SLE patients (n=5) that were cultured for 24 hr. As evident from the binding curves, a maximum inhibition in anti-TB antibody activity to the order of only 10.23 percent was observed (Fig. 49). Similarly, negligible inhibitions in anti-TB antibody activity of the order of 8.34 percent, 7.12 percent, 9.42 percent, 8.19 percent and 13.44 percent was observed with ds DNA isolated from control untreated, 10 mM NAC treated, 5 µg/ml EGCG treated, 100 µg/ml SN50 treated and 100 µg/ml SN50M treated monocytes respectively from SLE patients (Fig. 50).

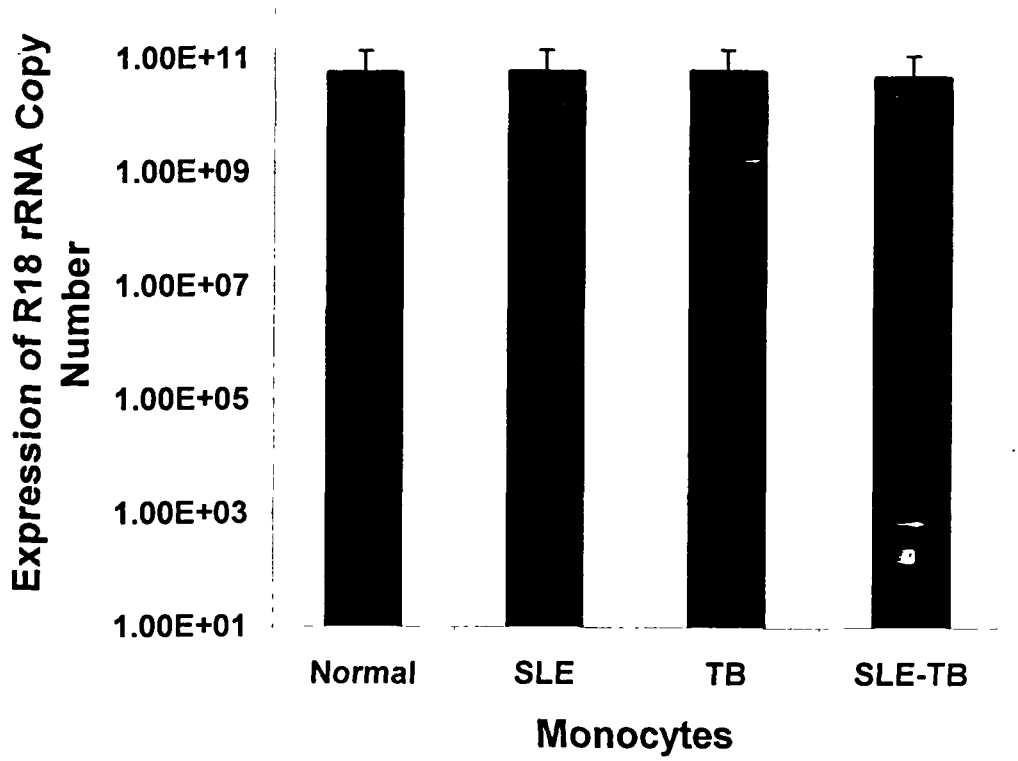


Fig. 1. Expression of human house keeping gene R18 rRNA in monocytes of patients with SLE (n=3; P<0.001), TB (n=3; P<0.001) and SLE-TB (n=3; P<0.001). Monocytes from normal healthy individuals served as controls (n=3). Data represents mean ± SEM of 3 experiments.

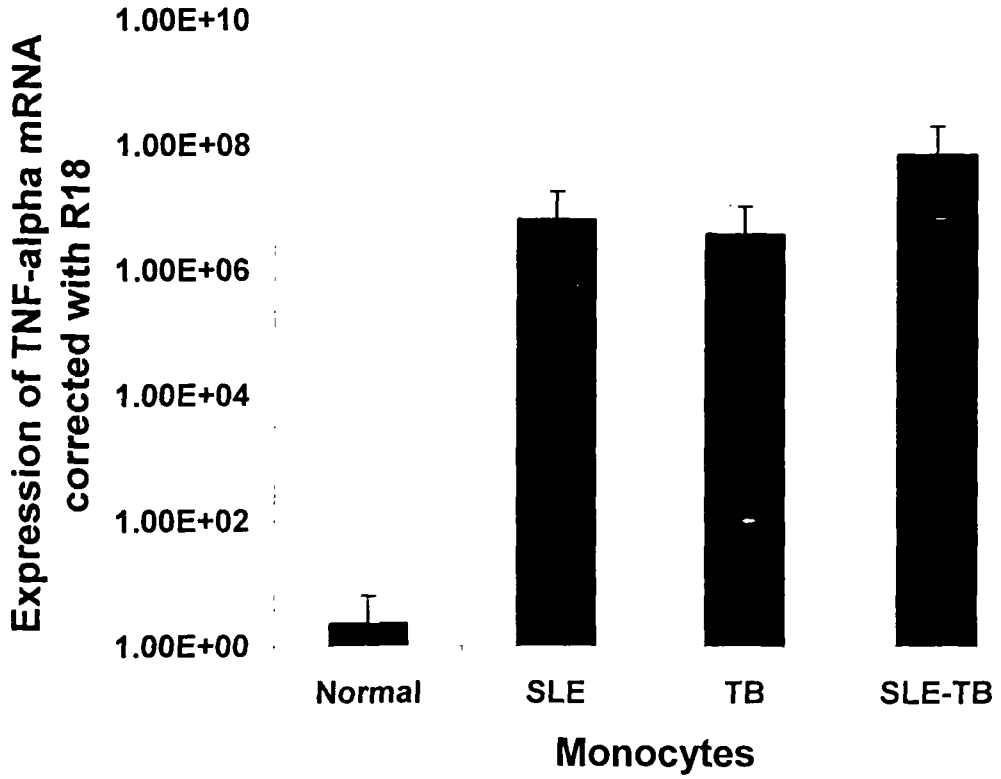


Fig. 2. Expression of human TNF-α mRNA in monocytes of patients with SLE (n=8; P<0.001), TB (n=8; P<0.001) and SLE-TB (n=8; P<0.001). Monocytes from normal healthy individuals served as controls (n=3). Data represents mean ± SEM of 8 experiments.

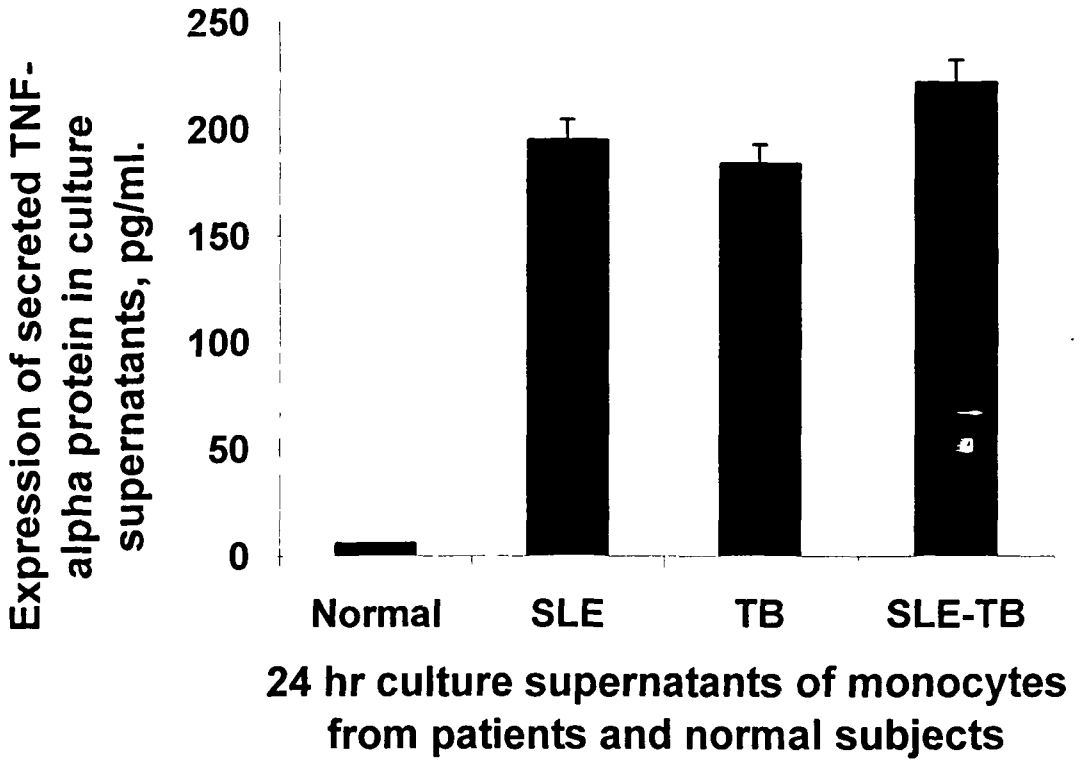


Fig. 3. Expression of secreted TNF-α protein in 24 hr culture supernatants of monocytes of patients with SLE (n=8; P<0.001), TB (n=8; P<0.001) and SLE-TB (n=8; P<0.001). Monocytes from normal healthy individuals served as controls (n=8). The concentration of secreted TNF- α was in pg/ml. Data represents mean ± SEM of 8 experiments.

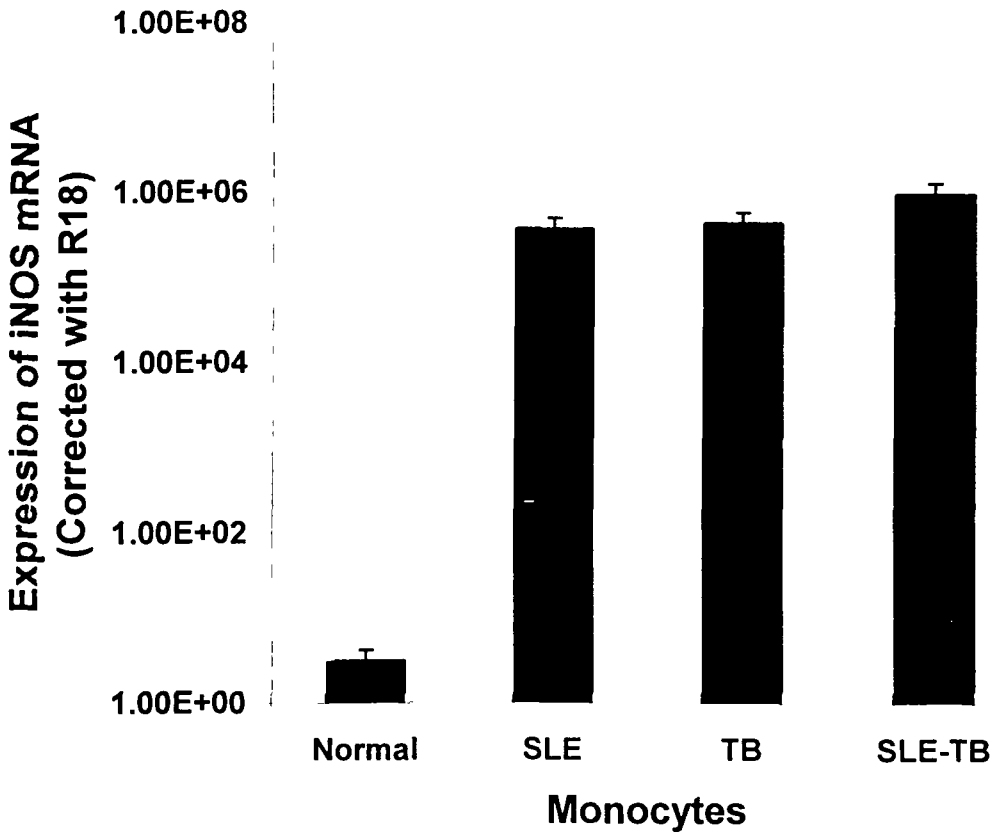


Fig. 4. Expression of iNOS mRNA in monocytes of patients with SLE (n=8; P<0.001), TB (n=8; P<0.001) and SLE-TB (n=8; P<0.001). Monocytes from normal healthy individuals served as controls (n=8). Data represents mean ± SEM of 8 experiments.

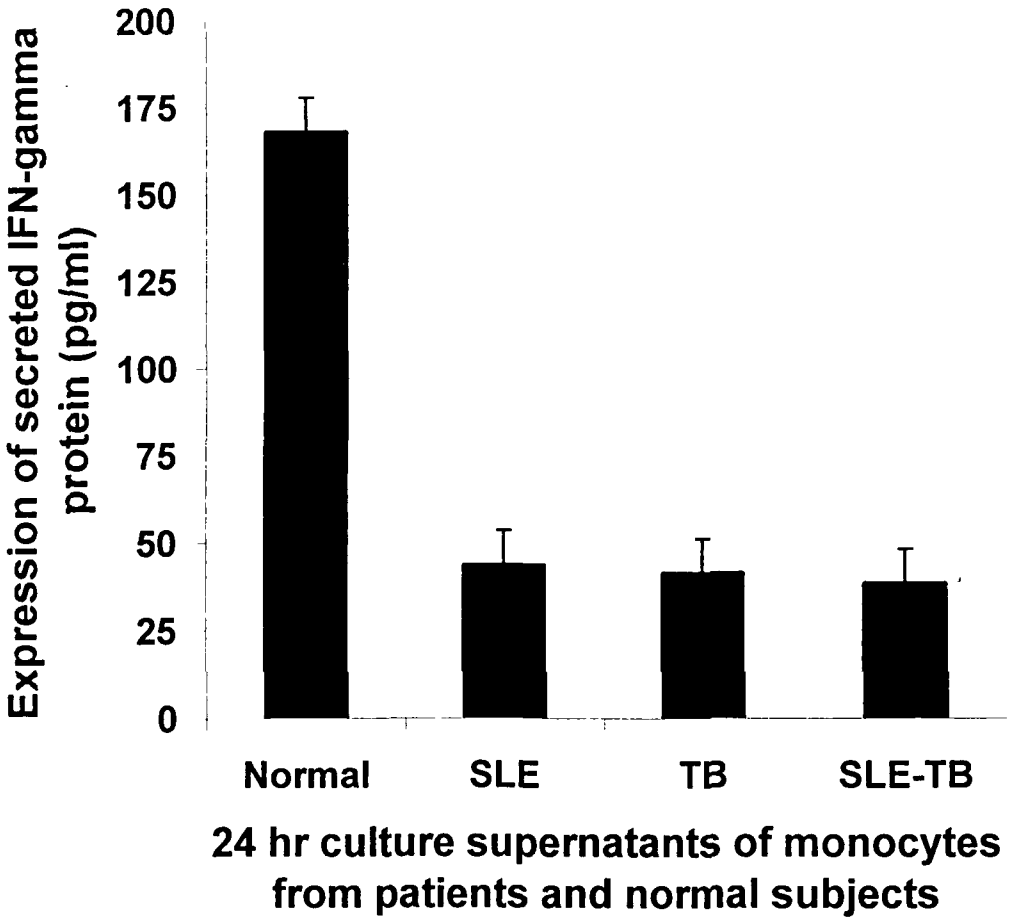


Fig. 5. Expression of secreted IFN-γ protein in 24 hr culture supernatants of monocytes of patients with SLE (n=8; P<0.001), TB (n=8; P<0.001) and SLE-TB (n=8; P<0.001). Monocytes from normal healthy individuals served as controls (n=8). The concentration of secreted IFN-γ was in pg/ml. Data represents mean ± SEM of 8 experiments.

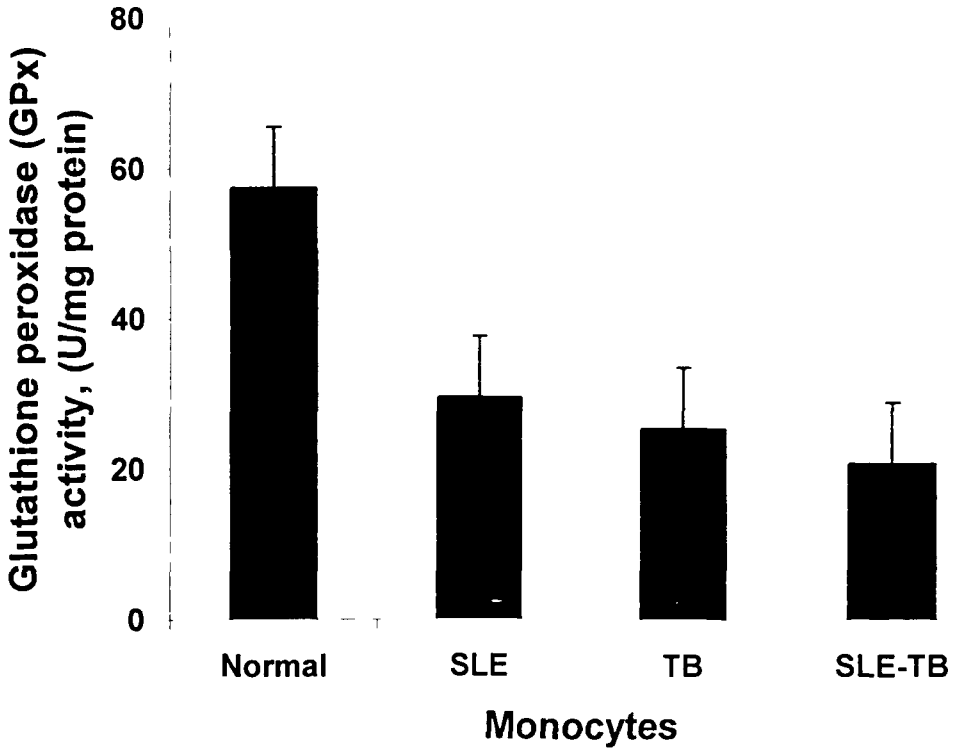


Fig. 6. Determination of glutathione peroxidase (GPx) activity in monocytes of patients with SLE (n=8; P<0.001), TB (n=8; P<0.001) and SLE-TB (n=8; P<0.001). Monocytes from normal healthy individuals served as controls (n=8). The unit of GPx activity was determined as U/mg protein. Data represents mean $\pm$ SEM of 8 experiments.

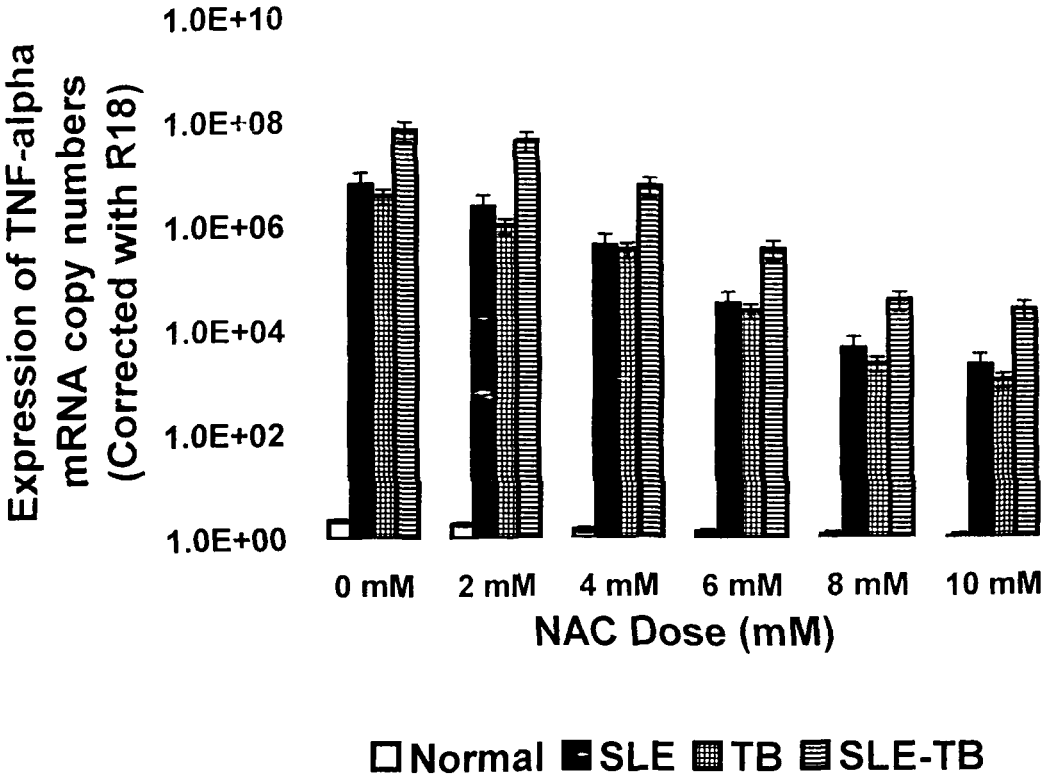


Fig. 7. Dose response effect of NAC (0-10 mM) on the expression of secreted TNF-α in 24 hr culture filtrates of monocytes of patients with SLE (black bars; n=3; $P<0.001$), TB (square bars; n=3; $P<0.001$) and SLE-TB (ladder bars; n=3; $P<0.001$). Monocytes from normal healthy individuals served as controls (empty bars; n=3). Data represents mean $\pm$ SEM of 8 experiments.

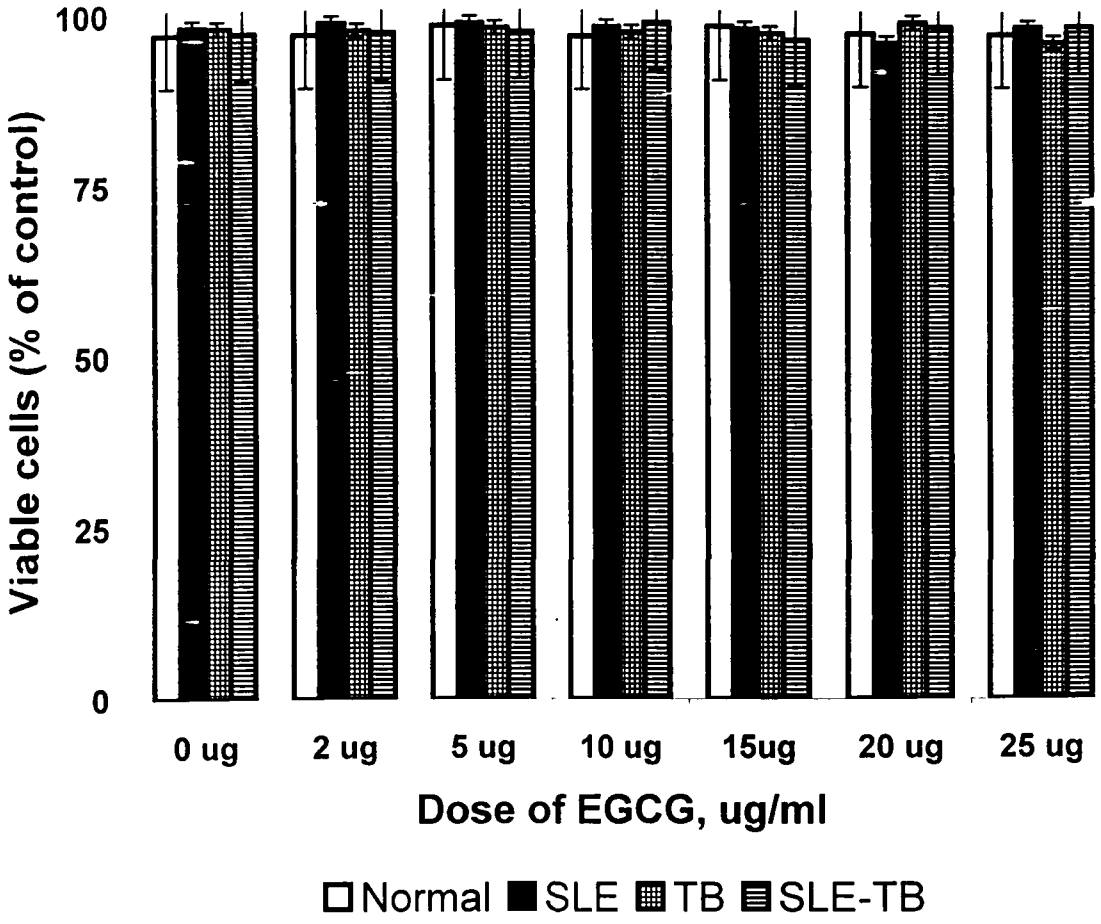


Fig 8: MTT cell viability assay for dose-response (0 – 25 ug/ml) effect of ECGC on 24 hr monocytes cultures of patients with SLE (black bars; n=3), TB (square bars; n=3), SLE-TB (ladder type bars; n=3) and normal healthy controls (empty bars; n=3). After 24 hrs of culture, the cells were harvested and processes as described in methods. Data represents the analysis of three independent experiments in duplicates, which are expressed as mean viable cells (±S. E.) percentage of controls. ($P<0.001$).

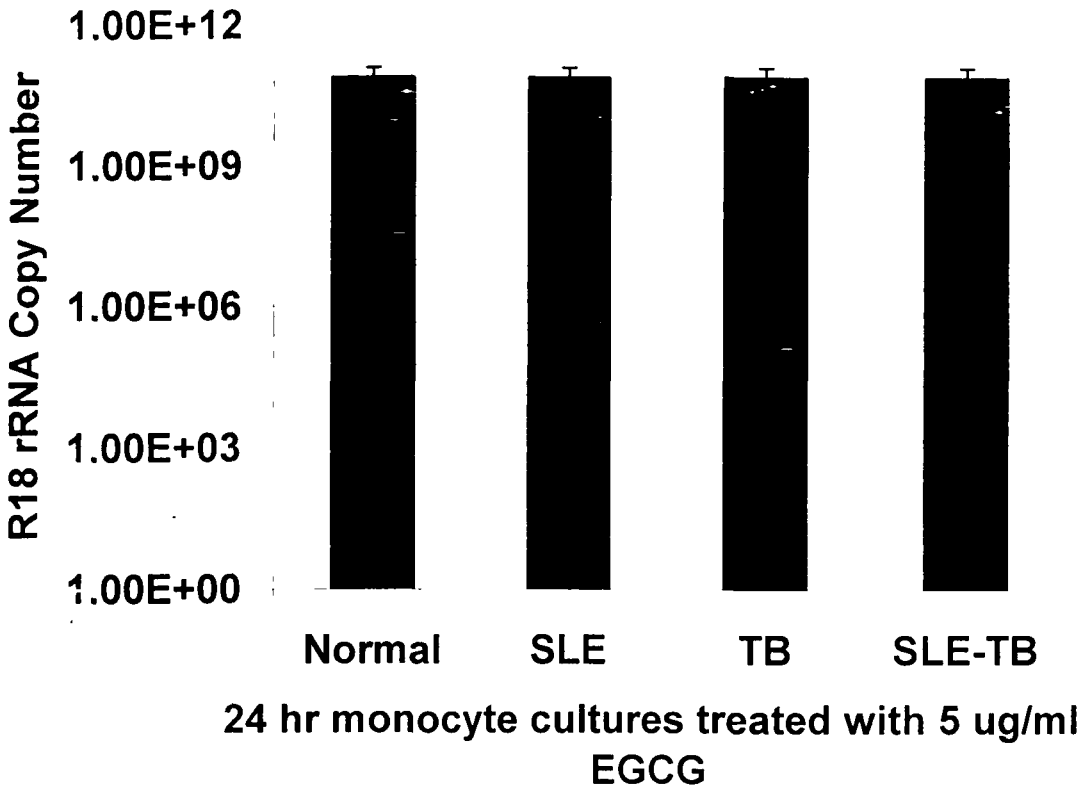


Fig. 9. Expression of human house keeping gene R18 r18 mRNA in 24 hr monocyte cultures treated with 5 µg/ml of EGCG. The monocytes were of patients with SLE (n=3; P<0.001)), TB (n=3; P<0.001) and SLE-TB (n=3; P<0.001). Monocytes from normal healthy individuals served as controls (n=3). Data represents mean ± SEM of 3 experiments.

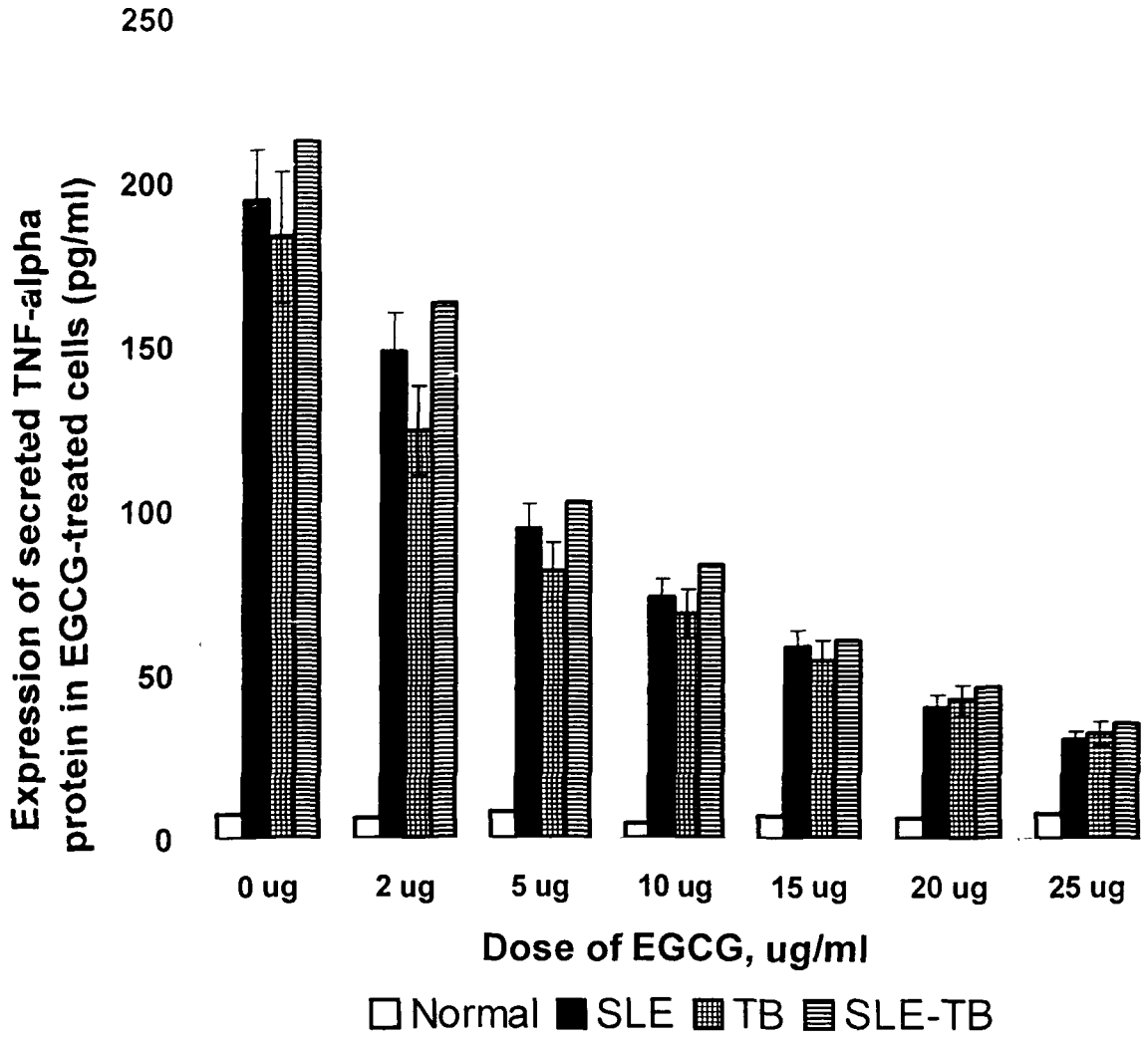


Fig. 10. Dose response effect of EGCG (0-25 µg/ml) on the expression of secreted TNF-α protein in 24 hr culture supernatants of monocytes of patients with SLE (black bars; n=5; P<0.001), TB (square bars; n=5; P<0.001) and SLE-TB (ladder bars; n=5; P<0.001). Monocytes from normal healthy individuals served as controls (empty bars; n=5; P<0.001). The concentration of secreted TNF- α was in pg/ml. IC_{50} was computed out to be < 5 ug/ml. Data represents mean ± SEM of 5 experiments.

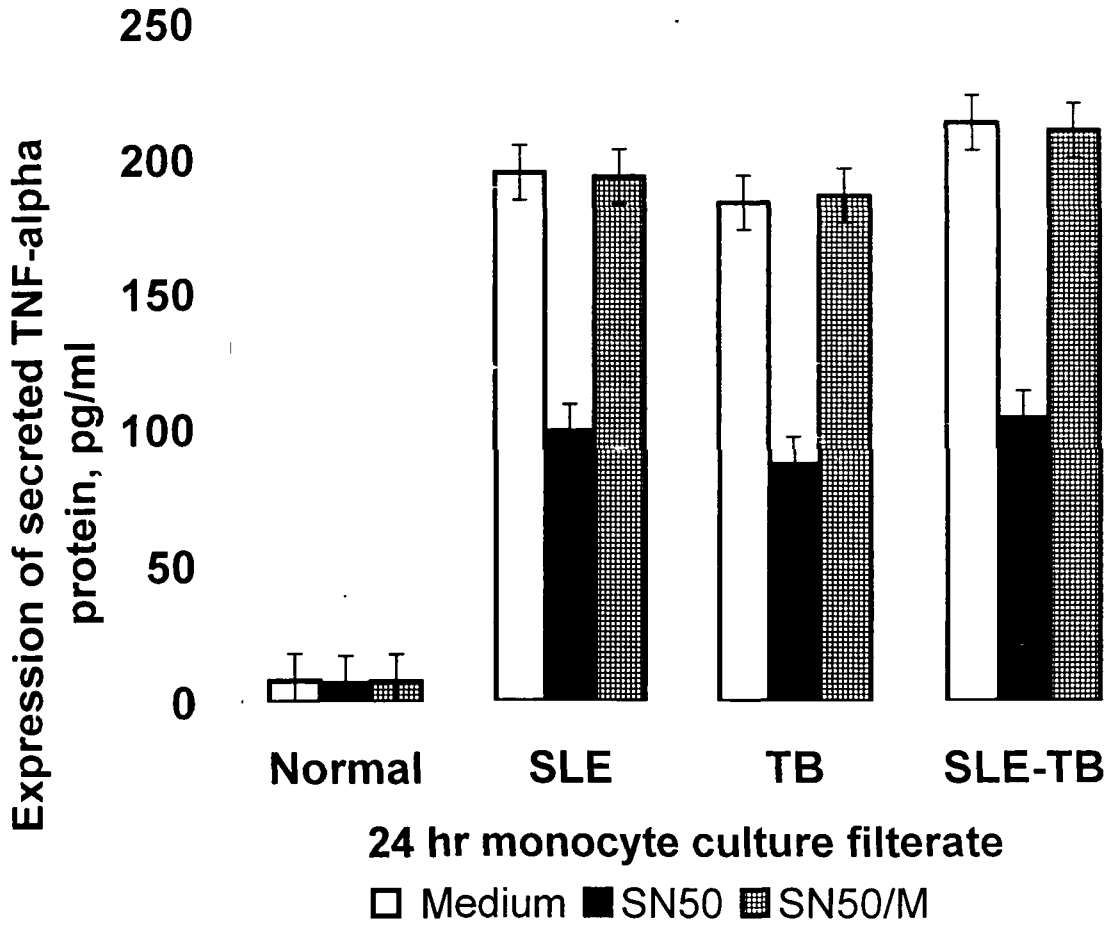


Fig. 11. Effect of SN50 (black bars; 100 µg/mlSN50M) and control peptide SN50M (square bars; 100 µg/ml) on the expression of secreted TNF-α protein in 24 hr culture supernatants of monocytes of patients with SLE (n=5; P<0.001), TB (n=5; P<0.001), SLE-TB (n=5; P<0.001) and normal healthy individuals (n=5). Untreated cultures served as controls (empty bars). The concentration of secreted TNF-α was in pg/ml. Data represents mean ± SEM of 5 experiments.

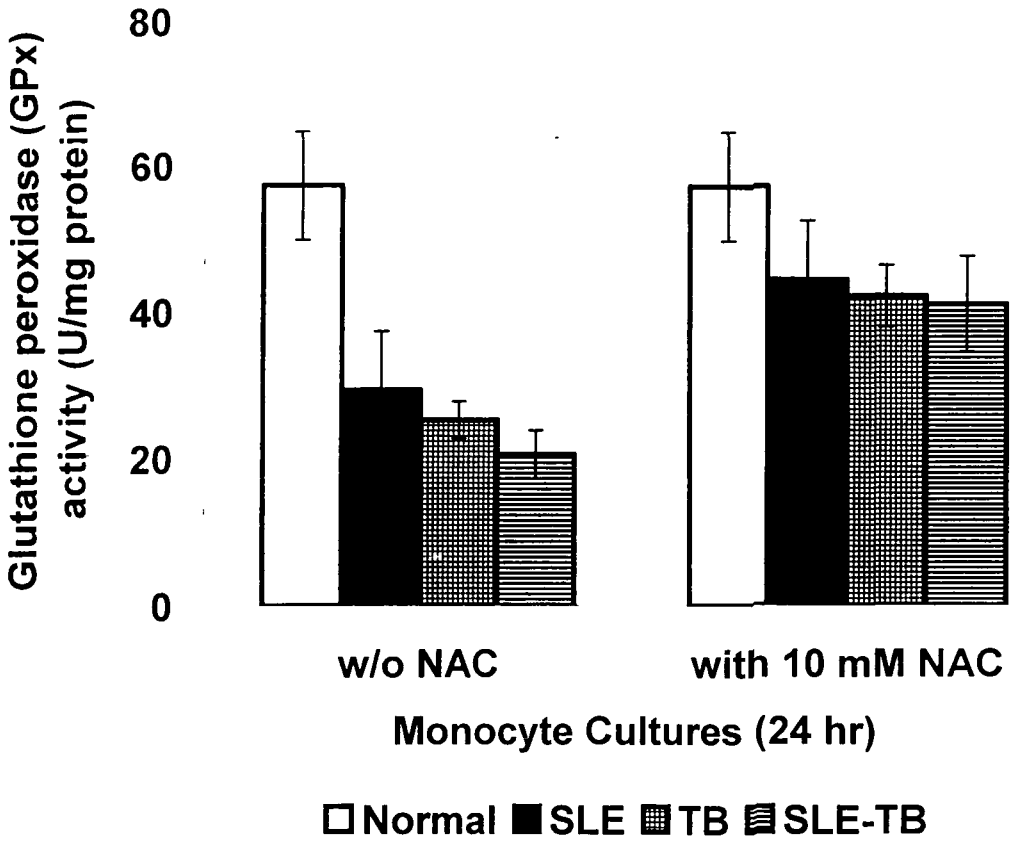


Fig. 12. NAC (10 mM)-induced modulation of glutathione peroxidase (GPx) activity (U/mg protein) in 24 hr monocytes cultures of patients with SLE (black bars; n=8; $P<0.001$), TB (square bars; n=8; $P<0.001$) and SLE-TB (ladder bars; n=8; $P<0.001$). Monocytes from normal healthy individuals served as controls (empty bars; n=8). Data represents mean $\pm$ SEM of 8 experiments.

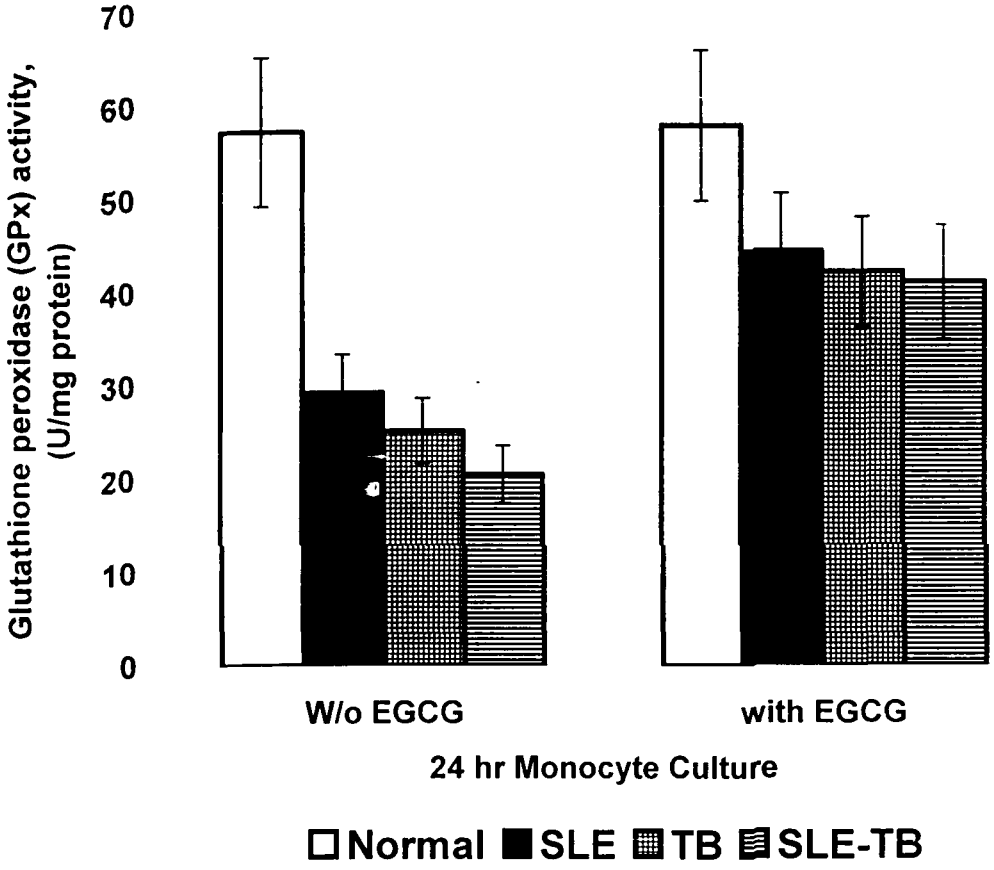


Fig. 13. EGCG (5 µg/ml)-induced modulation of glutathione peroxidase (GPx) activity (U/mg protein) in 24 hr monocytes cultures of patients with SLE (black bars; n=8; $P<0.001$), TB (square bars; n=8; $P<0.001$) and SLE-TB (ladder bars; n=8; $P<0.001$). Monocytes from normal healthy individuals served as controls (empty bars; n=8). Data represents mean $\pm$ SEM of 8 experiments.

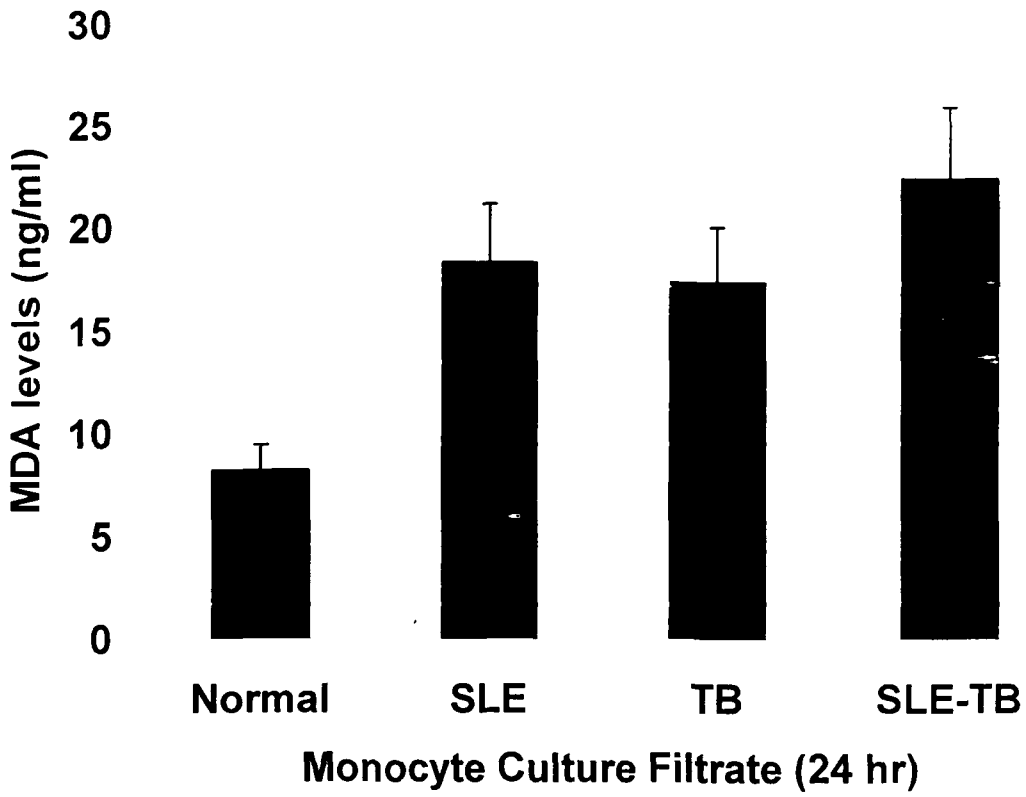


Fig. 14. Expression MDA in 24 hr culture supernatants of monocytes of patients with SLE (n=3; P<0.001), TB (n=3; P<0.001) and SLE-TB (n=3; P<0.001). Monocytes from normal healthy individuals served as controls (n=3). The concentration of MDA measured was in pg/ml. Data represents mean $\pm$ SEM of 3 experiments.

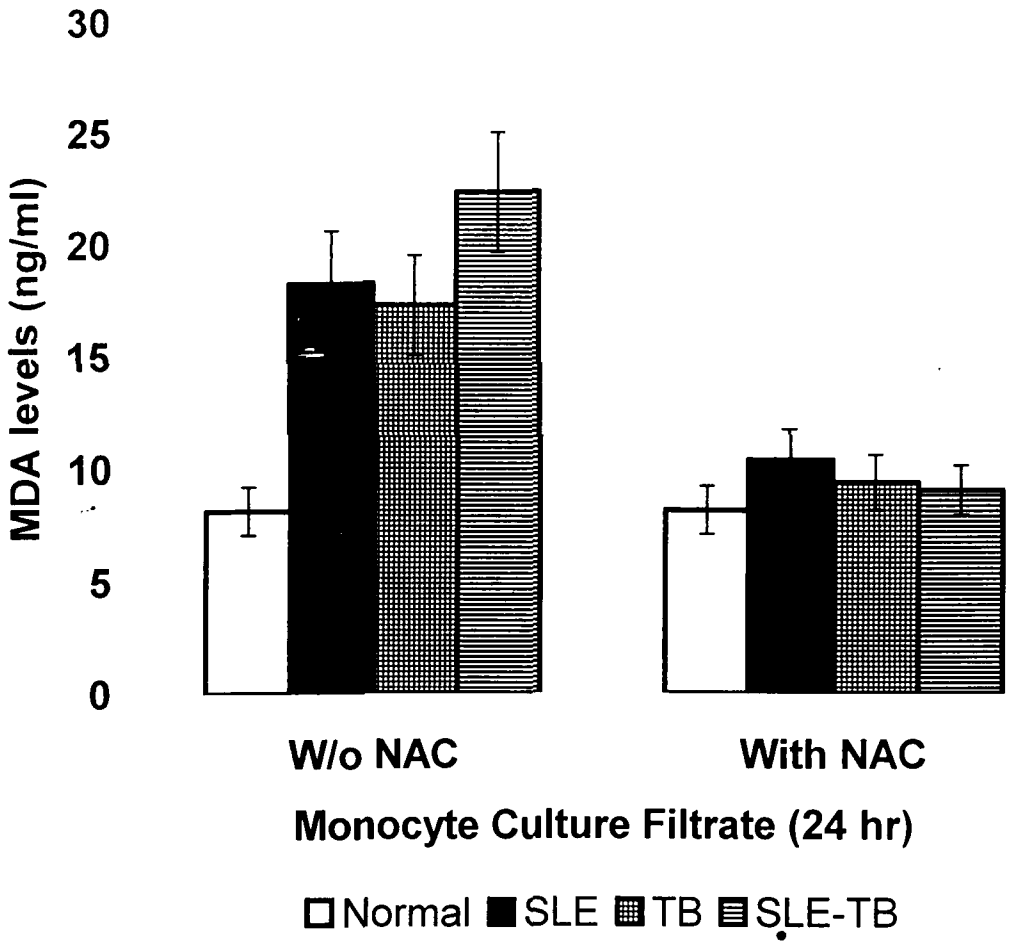


Fig. 15. Modulation of the expression MDA levels by 10 mM NAC in 24 hr culture supernatants of monocytes of patients with SLE (n=3; P<0.001), TB (n=3; P<0.001) and SLE-TB (n=3; P<0.001). Monocytes from normal healthy individuals served as controls (n=3; P<0.001). The concentration of MDA measured was in pg/ml. Data represents mean ± SEM of 3 experiments.

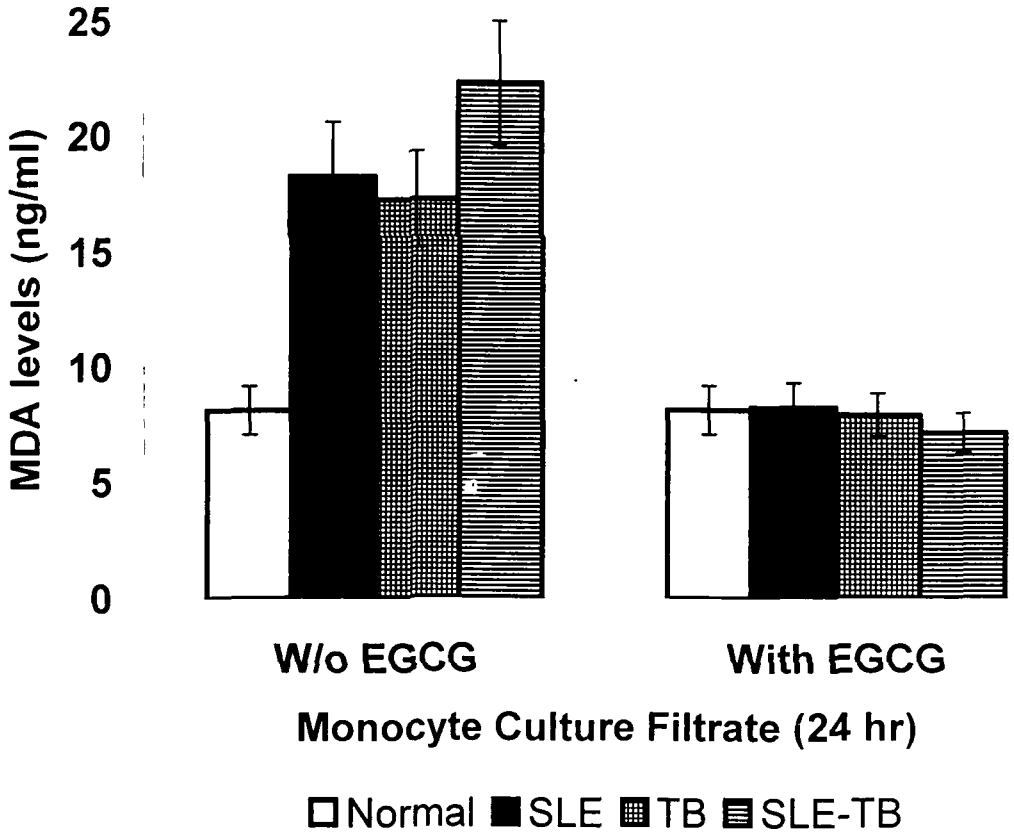


Fig. 16. Modulation of the expression MDA levels by 5 ug/ml EGCG in 24 hr culture supernatants of monocytes of patients with SLE (n=3; P<0.001), TB (n=3; P<0.001) and SLE-TB (n=3; P<0.001). Monocytes from normal healthy individuals served as controls (n=3). The concentration of MDA measured was in pg/ml. Data represents mean ± SEM of 3 experiments.

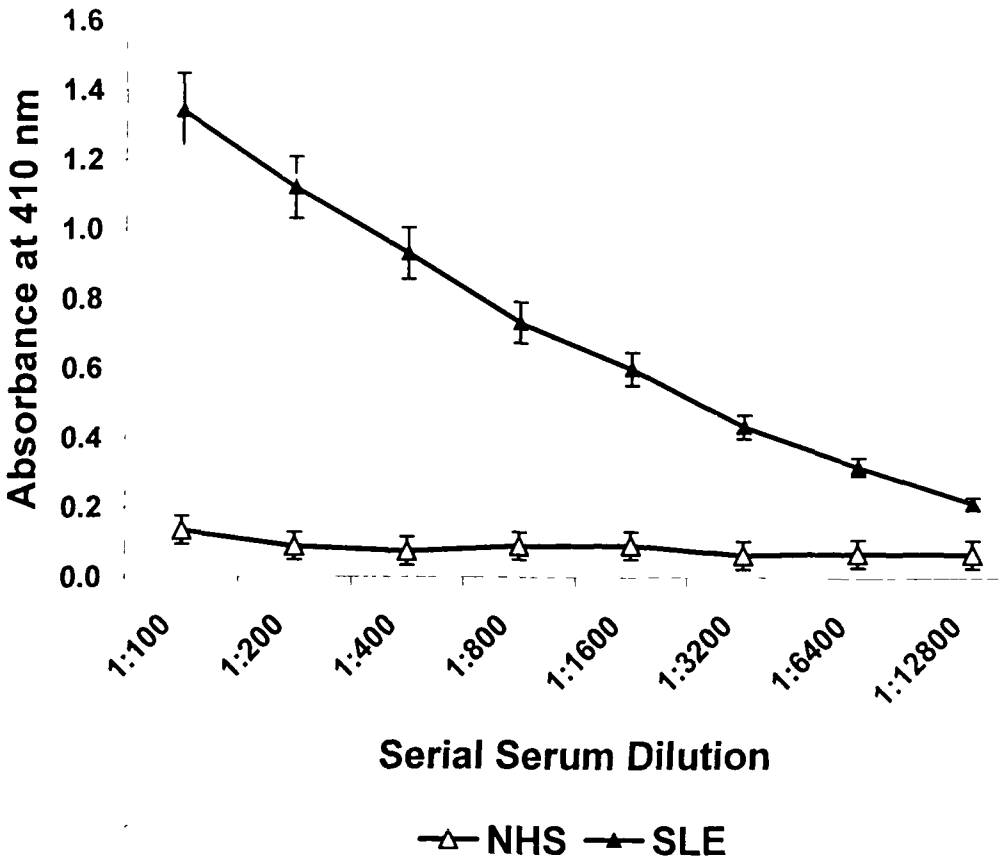


Fig. 17. Direct binding ELISA on microtitre ELISA plates that were coated with purified calf thymus dsDNA (100 ul/well from 250 ng/ml stock) against anti-DNA antibodies (-▲- ; SLE sera; n=20; P<0.001). Normal human sera served as controls (-Δ-; NHS) Data represents mean ± SEM of number of experiments.

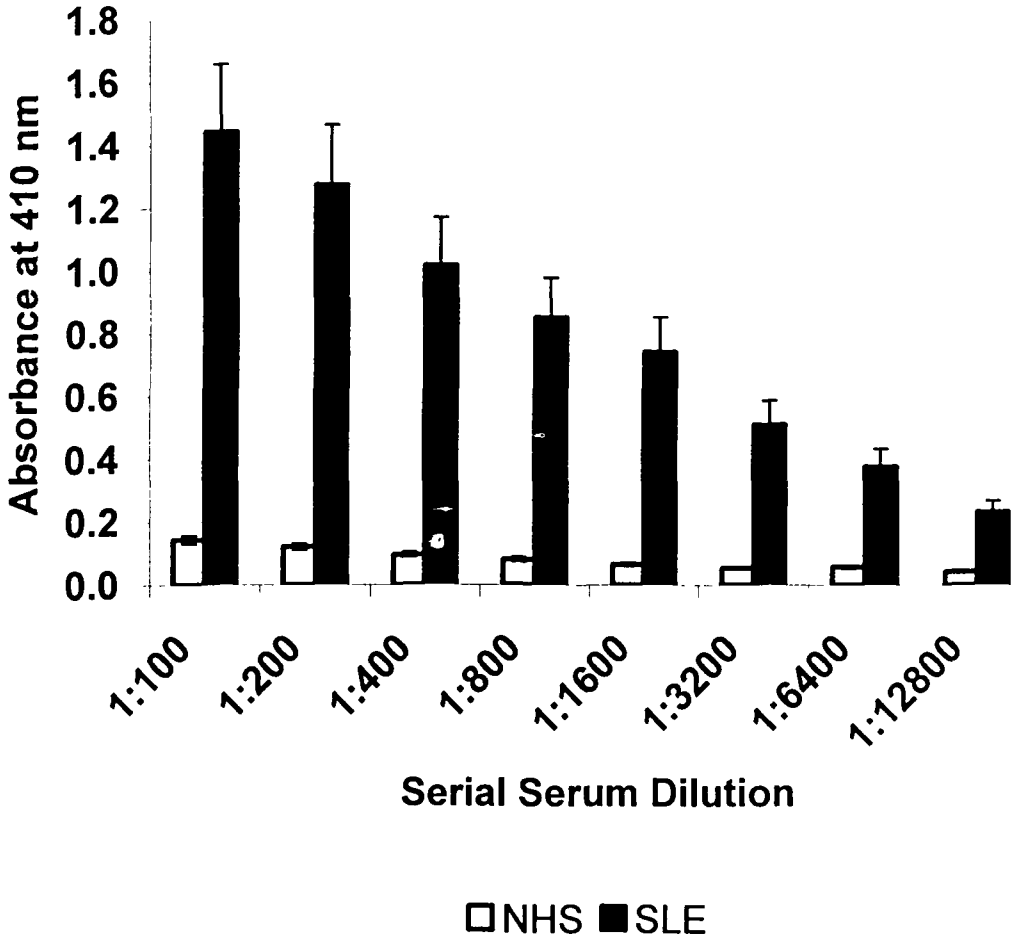


Fig. 18. Direct binding ELISA on microtitre ELISA plates that were coated with Mycobacterial total sonic extract (100 ul/well from 20 ug/ml stock) against anti-DNA antibodies (-▲-; SLE sera; n=20; P<0.001). Normal human sera served as controls (-Δ-; NHS) Data represents mean ± SEM of number of experiments.

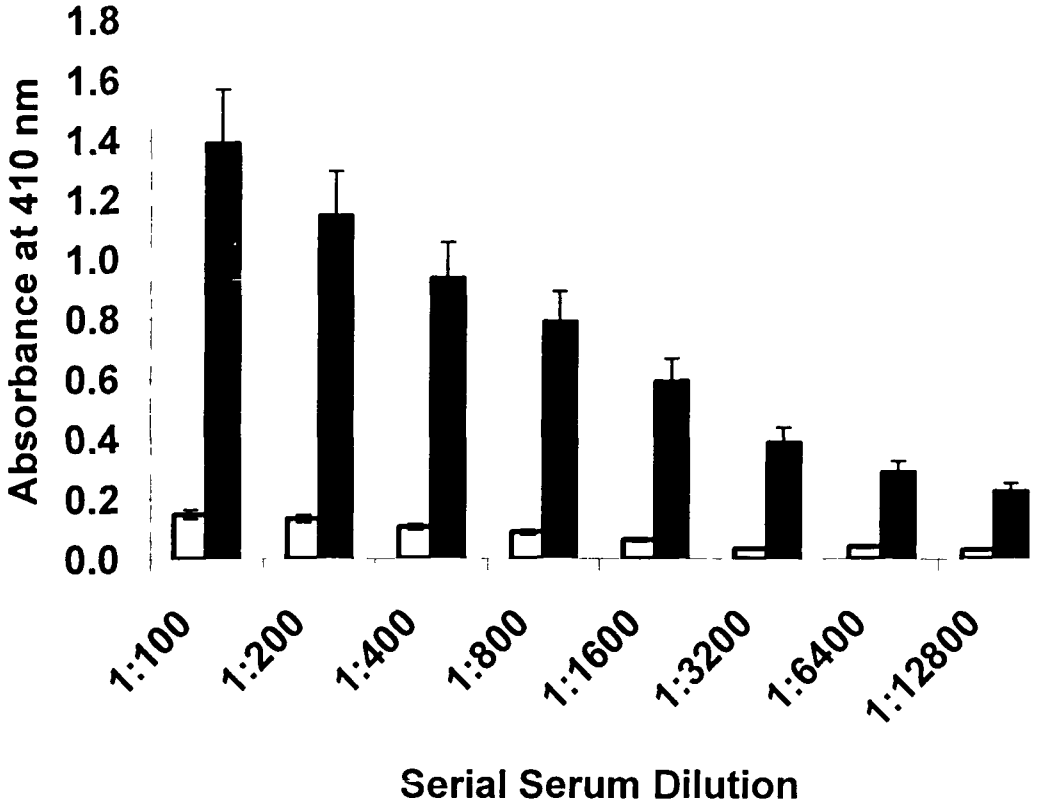


Fig. 19. Direct binding ELISA on microtitre ELISA plates that were coated with Mycobacterial total culture filtrate (MTCF) (100 µl/well from 20 ug/ml stock) against anti-DNA antibodies (-▲- ; SLE sera; n=20; P<0.001). Normal human sera served as controls (-Δ-;NHS; n=10). Data represents mean ± SEM of number of experiments.

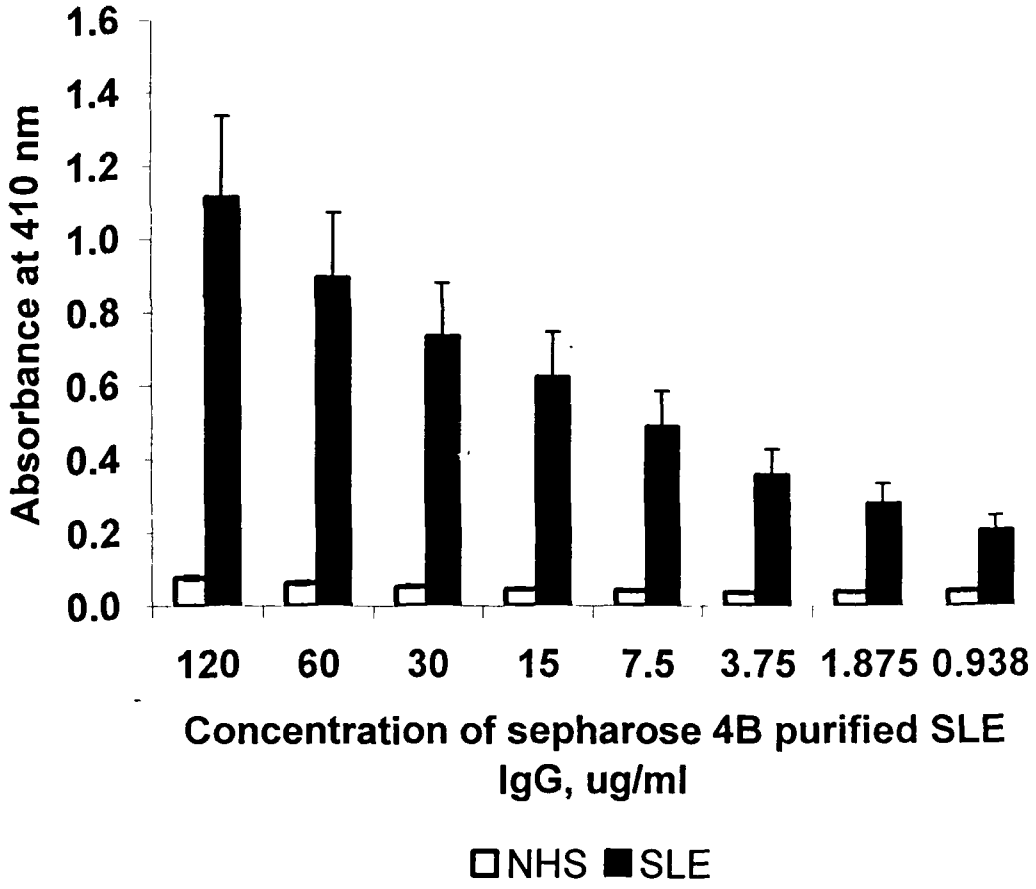


Fig. 20. Direct binding ELISA on microtitre ELISA plates that were coated with Mycobacterial total culture filtrate (MTCF) (100 µl/well from 20 ug/ml stock) against Sepharose 4B purified SLE IgG i. e. purified anti-DNA antibodies (-▲-; IgG from SLE sera; n=4; $P<0.001$). Normal human sera served as controls (-Δ-; IgG from NHS; n=4). Data represents mean ± SEM of 4 experiments.

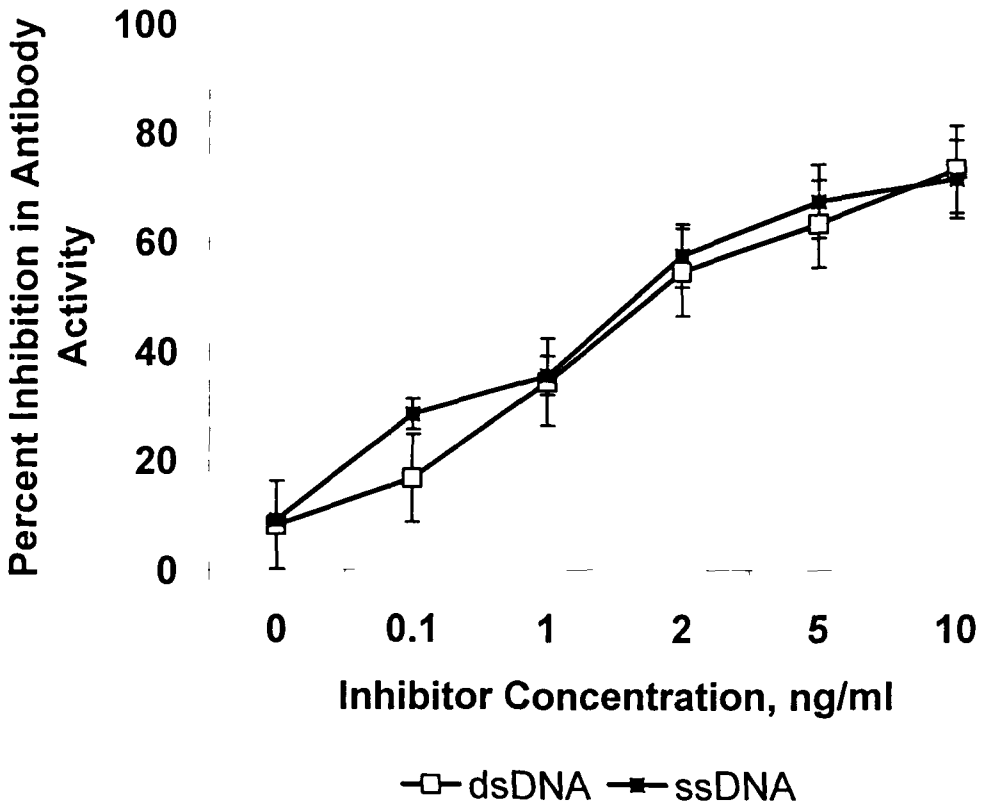


Fig. 21. Inhibition ELISA on microtitre ELISA plates that were coated with dsDNA (∓) (100 ul/well from 2.5 ng/ml stock) and ssDNA (-■-) (100 ul/well from 2.5 ng/ml stock) against Sepharose 4B purified SLE IgG i. e. purified anti-DNA antibodies (n=4; $P<0.001$). The inhibitors were dsDNA and ssDNA. The immune complex prepared was used in place of antibody as described in methods. Fifty percent inhibition in anti-DNA antibody activity (IC_{50}) for dsDNA and ssDNA was computed out to be 2.2 µg/ml and 1.2 ug/ml respectively. Data represents mean ± SEM of 4 experiments.

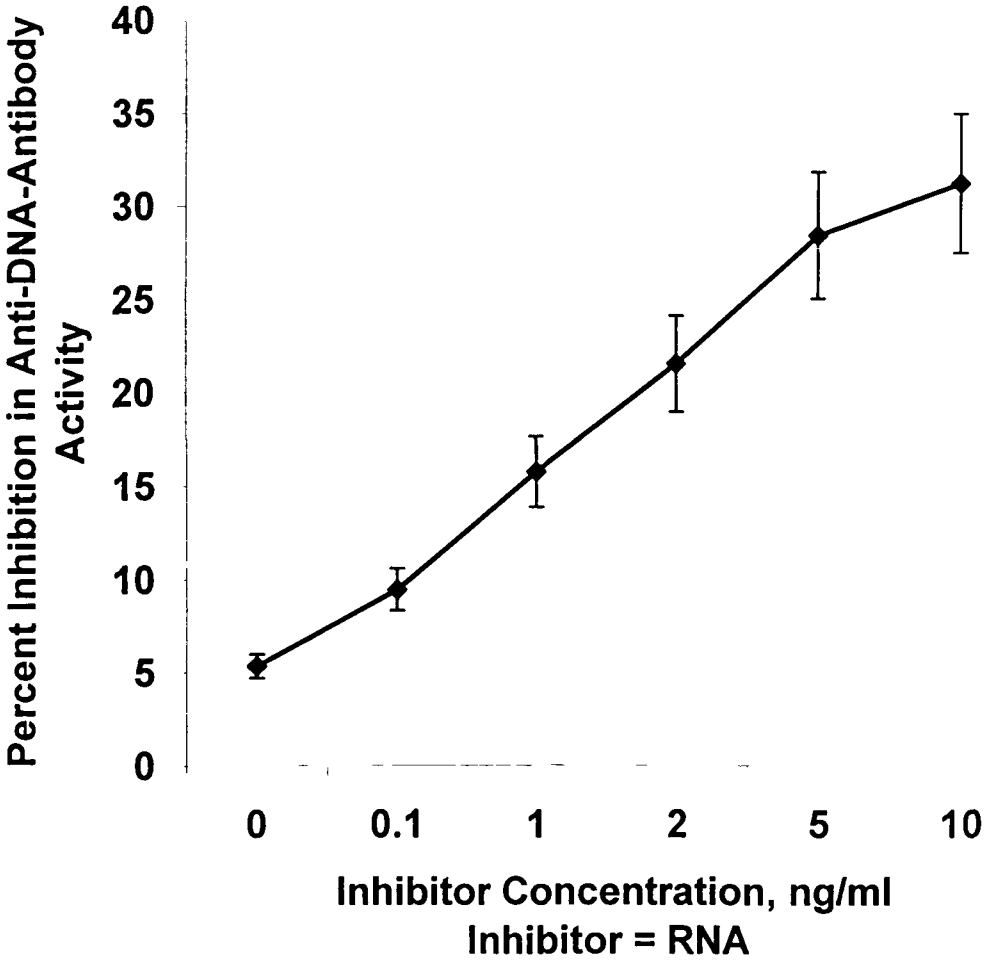


Fig. 22. Inhibition ELISA for RNA on microtitre ELISA plates that were coated with dsDNA (-■-) (100 ul/well from 2.5 ng/ml stock) against Sepharose 4B purified SLE IgG i. e. purified anti-DNA antibodies (n=4; $P<0.001$). The inhibitor used to prepare immune complex was RNA. The immune complex was used in place of antibody as described in methods. Fifty percent inhibition in antibody activity (IC_{50}) could not be achieved. Data represents mean $\pm$ SEM of 4 experiments.

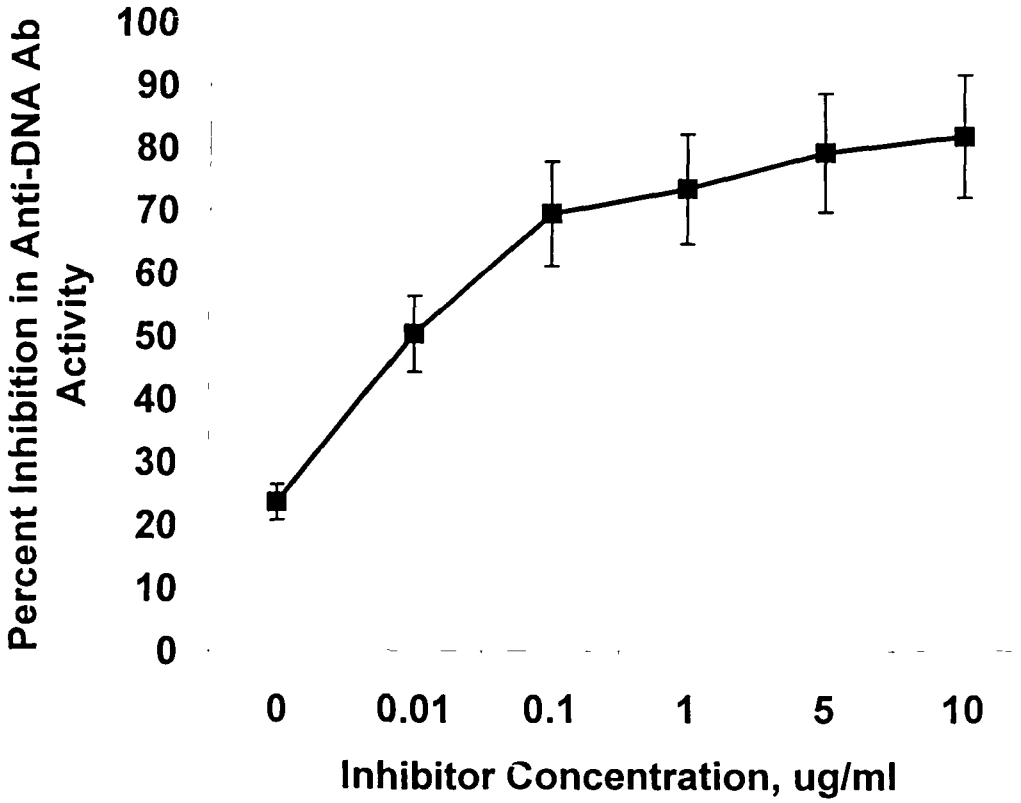


Fig. 23. Inhibition ELISA for MTCF on microtitre ELISA plates that were coated with dsDNA (100 µl/well from 2.5 ng/ml stock) against Sepharose 4B purified SLE IgG i. e. purified anti-DNA antibodies (n=4; $P<0.001$). The inhibitor was MTCF. The immune complex prepared with varying doses of MTCF was used in place of antibody as described in methods. Fifty percent inhibition in anti-DNA antibody activity (IC_{50}) was computed out to be 0.01 ug/ml. (MTCF= Mycobacterial total culture filtrate i. e. secretory proteins). Data represents mean $\pm$ SEM of 4 experiments.

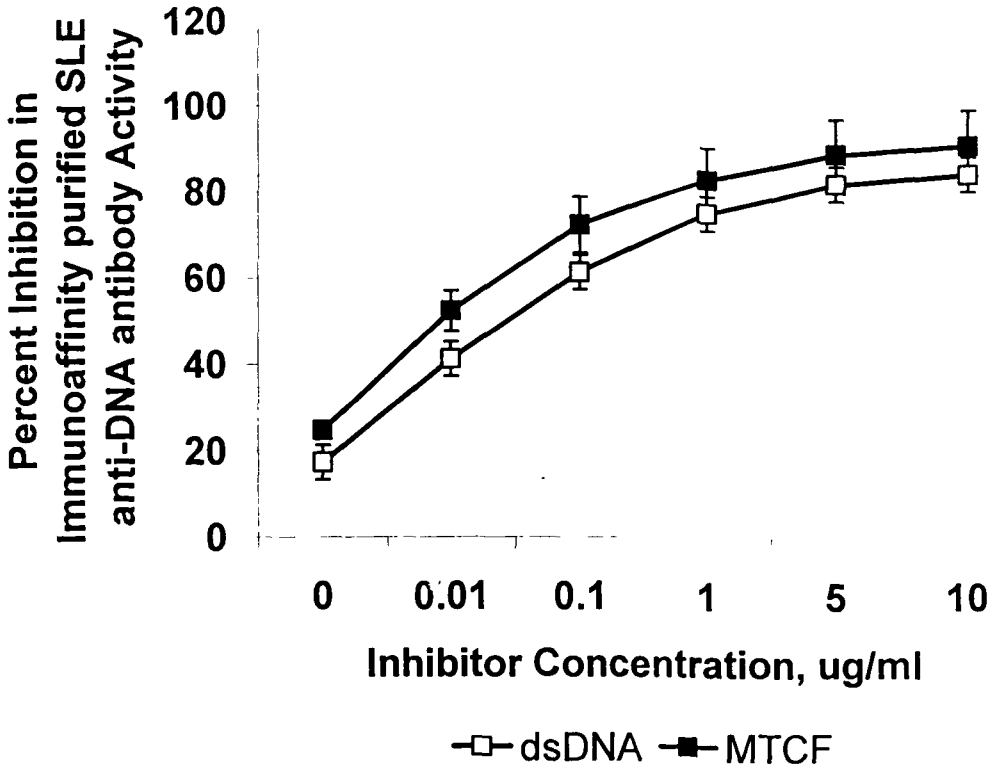


Fig. 24. Inhibition ELISA for MTCF on microtitre ELISA plates that was coated with dsDNA (100 ul/well from 2.5 ng/ml stock) against immunoaffinity purified anti-DNA antibodies (on DNA-polylysyl-sepharose 4B column) i. e. immunoaffinty purified SLE IgG (n=4; $P<0.001$). The inhibitor was dsDNA (-□-) and MTCF (-■-). The immune complex prepared with varying doses of dsDNA or MTCF, and was used in place of antibody as described in methods. Fifty percent inhibition in anti-DNA antibody activity (IC_{50}) was computed out to be 0.01 ug/ml for both dsDNA and MTCF. (MTCF= Mycobacterial total culture filtrate i. e. secretory proteins). Data represents mean ± SEM of 4 experiments.

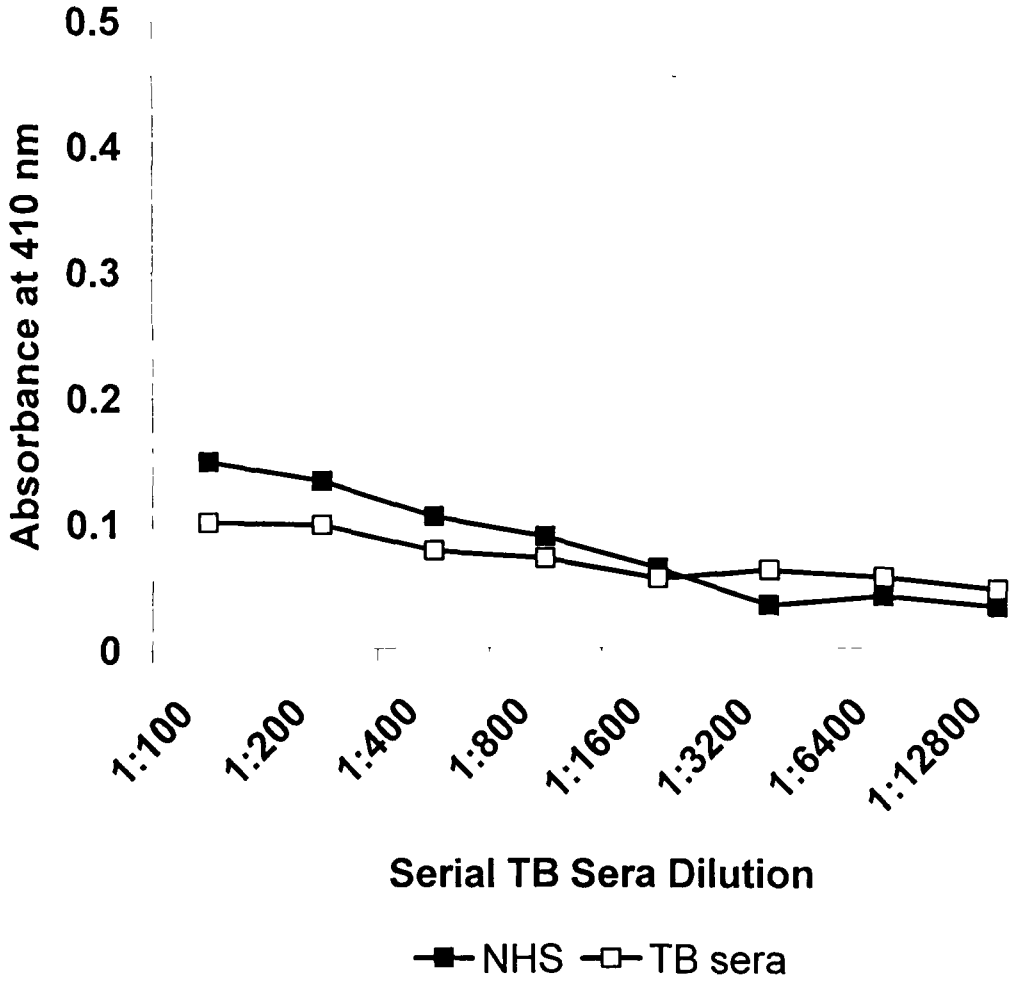


Fig. 25. Direct binding ELISA on microtitre ELISA plates that were coated with dsDNA (100 µl/well from 2.5 ng/ml stock) against TB antibodies (TB sera) (-□-). Normal human sera served as controls (-■-NHS). No binding was observed, as the titer was <1:100. Data represents mean ± SEM of 8 experiments.

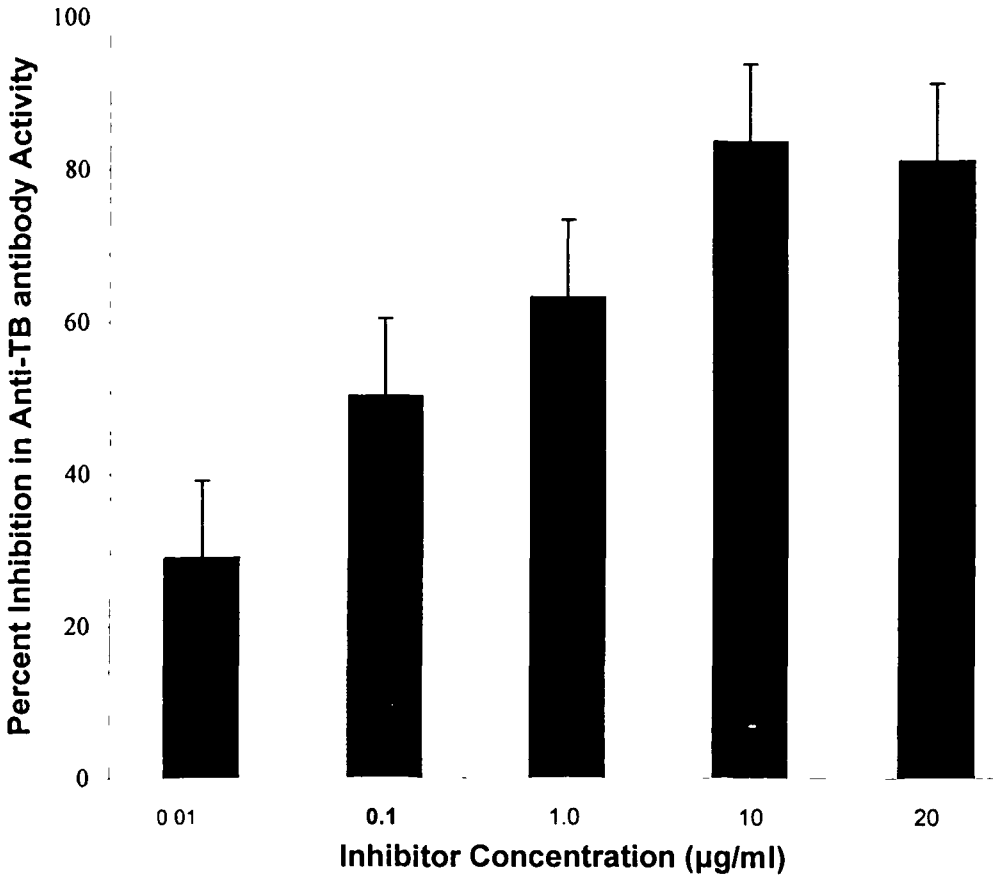


Fig. 26. Inhibition ELISA against antibodies in sera of patients with TB (n=30) on plates coated with 30 kDa Ag85B isolated from mid-logarithmic phase cultures of *Mycobacterium tuberculosis* $H_{37}Rv$. The inhibitor used here was varying concentrations of 30 kDa Ag85B. The concentrations were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data are mean ±SD.

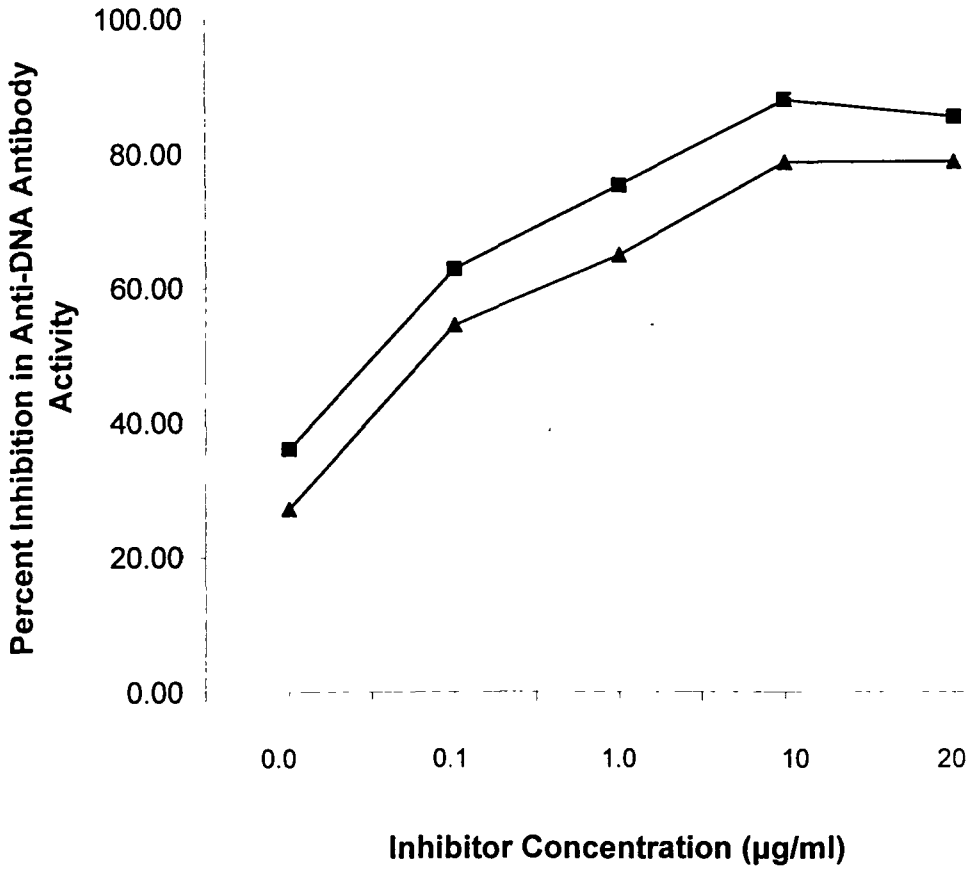


Fig. 27. Comparative Inhibition ELISA against autoantibodies in Patients with SLE (n=30) on plates coated with native dsDNA (non mycobacterial), where the inhibitors used were varying concentrations of native dsDNA versus mycobacterial 30 kDa Ag85B.The concentrations for both the inhibitors were 0.01, 0.1, 1.0, 10 and 20 µg/ml. dsDNA (-■-) and mycobacterial 30kDa Ag85B (-▲). Data are mean ±SD.

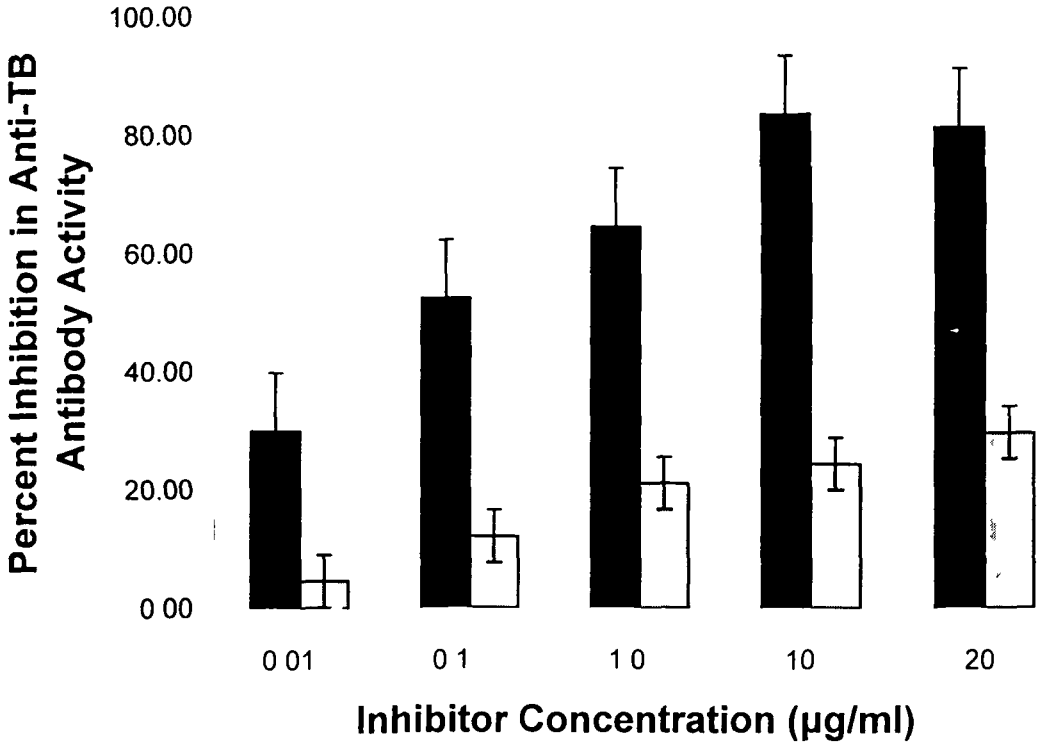


Fig. 28. Comparative Inhibition ELISA against antibodies in patients with TB (n=30) on plates coated with mycobacterial 30 kDa Ag85B, where the inhibitors used were varying concentrations of mycobacterial 30 kDa Ag85B versus non-mycobacterial native dsDNA. The concentrations for both the inhibitors were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Mycobacterial 30 kDa (Black bars) and non-mycobacterial native dsDNA (shaded bars). Data are mean ±SD.

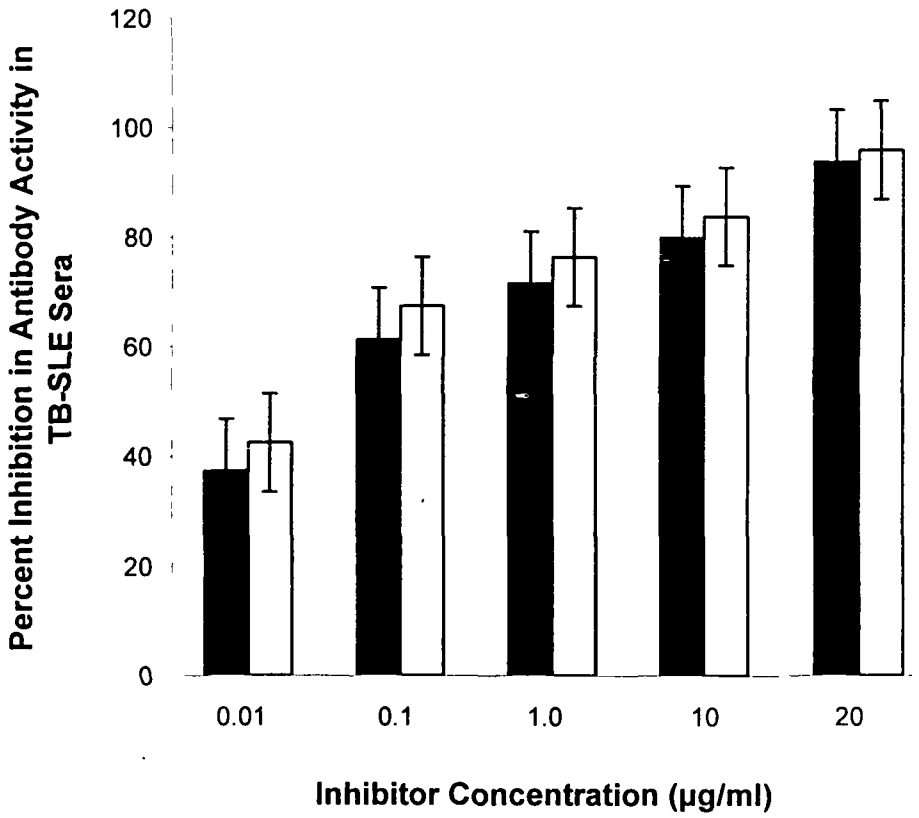


Fig. 29. Comparative Inhibition ELISA against antibodies in patients with TB-SLE (n=30) on plates coated with mycobacterial 30 kDa Ag85B, where the inhibitors used were varying concentrations of mycobacterial 30 kDa Ag85B versus non-mycobacterial native dsDNA. The concentrations for both the inhibitors were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Mycobacterial 30 kDa Ag85B (Black bars) and non mycobacterial native dsDNA (white bars). Data are mean ±SD.

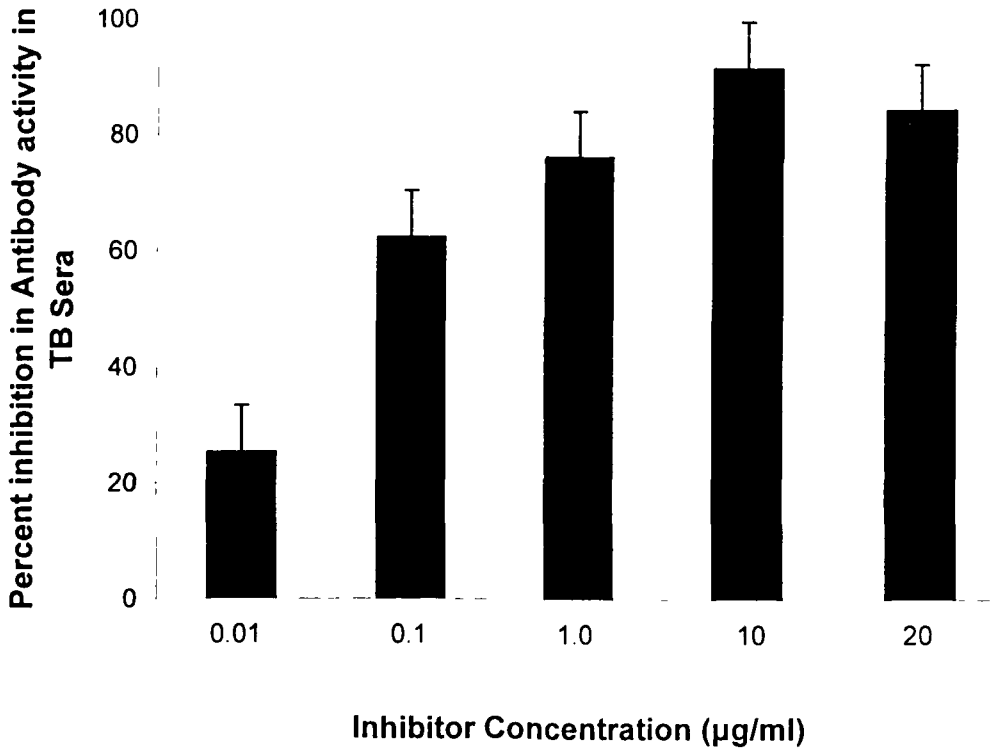


Fig. 30. Inhibition ELISA against antibodies in sera of patients with TB (n=5) on plates coated with total protein lysates obtained from human monocytes isolated from blood of TB patients where the inhibitor used were varying concentrations of the above mentioned total protein lysates obtained from monocytes of TB patients. The inhibitor concentrations were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data are mean ±SD.

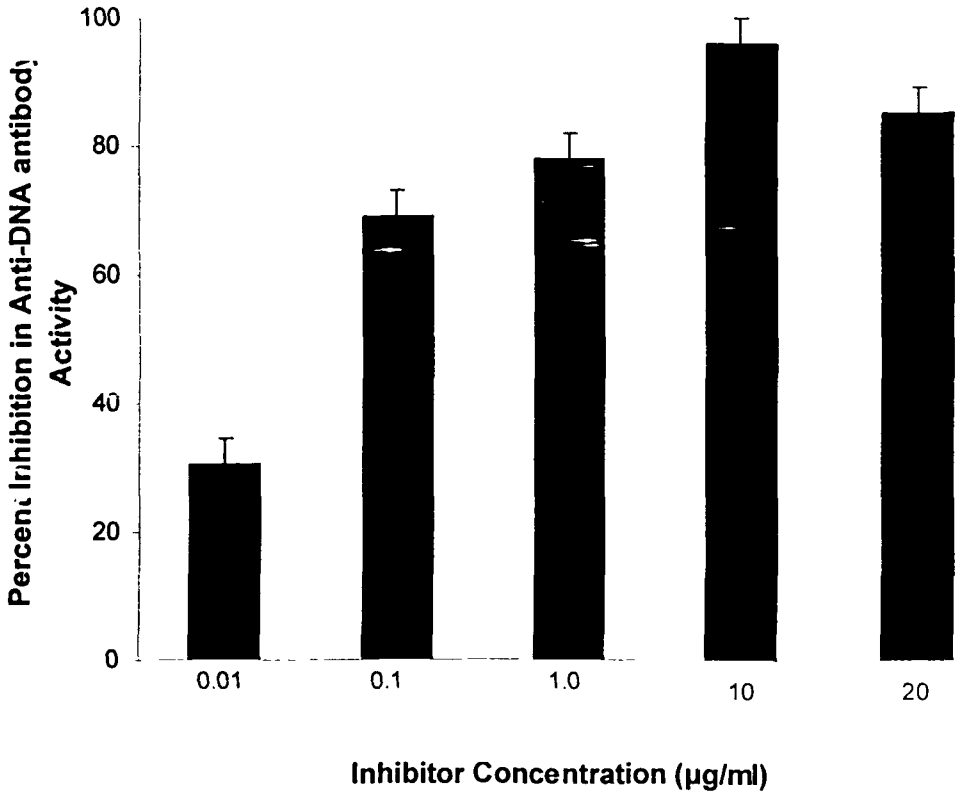


Fig. 31. Inhibition ELISA against antibodies in sera of patients with SLE (n=5) on plates coated with non-mycobacterial native dsDNA and the inhibitors used were lysates obtained from monocytes isolated from blood of SLE patients where the varying inhibitor concentrations were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data are mean ±SD.

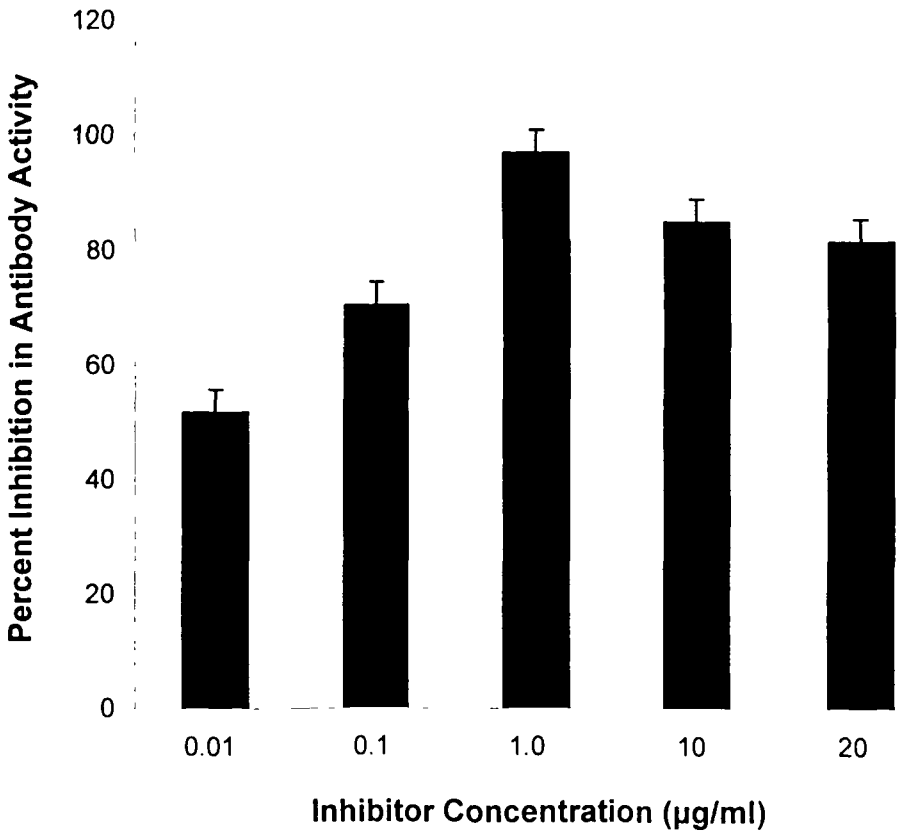


Fig. 32. Inhibition ELISA against antibodies in sera of patients with TB-SLE (n=5) on plates coated with non-mycobacterial native dsDNA and the inhibitors used were lysates obtained from monocytes isolated from blood of SLE patients where the varying inhibitor concentrations were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

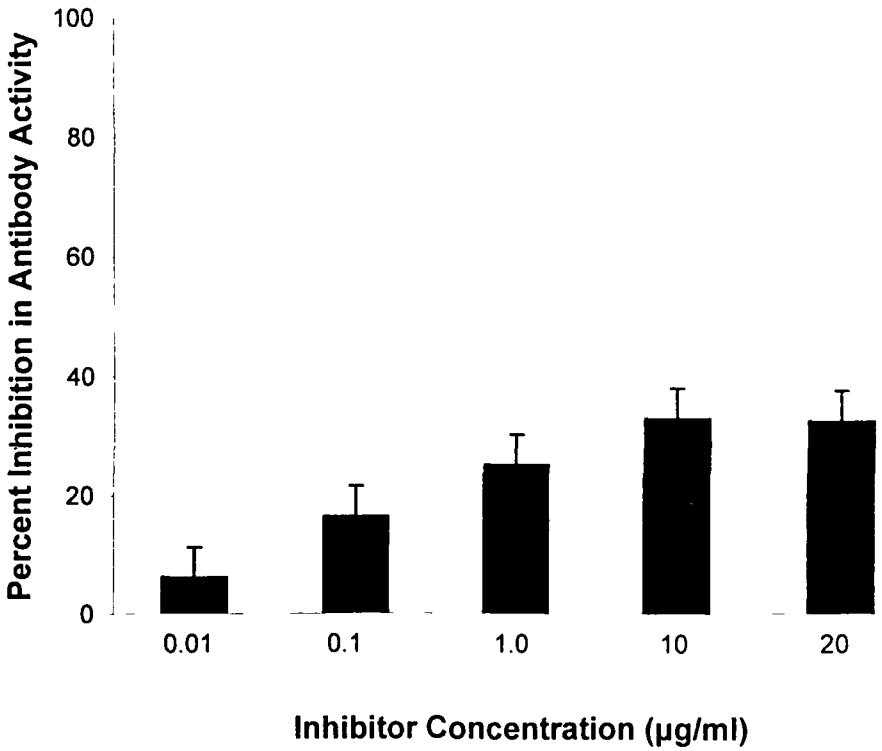


Fig. 33. Modulation of TB monocytes with reduced glutathione: Inhibition ELISA against antibodies in sera of patients with TB (n=5) on plates coated with total protein lysates obtained from TB patient monocytes that were co-cultured for 24 hrs with 10 nM of reduced glutathione where the varying inhibitor concentrations used of the above mentioned total protein lysates were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

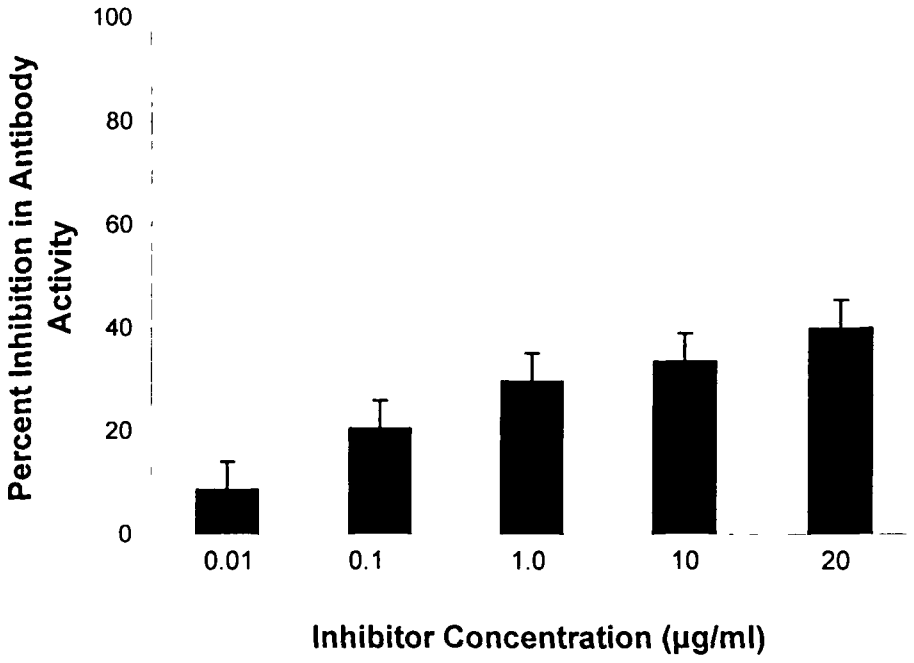


Fig. 34. Modulation of SLE monocytes with reduced glutathione: Inhibition ELISA against antibodies in sera of patients with SLE (n=5) on plates coated with total protein lysates obtained from SLE patient monocytes that were co-cultured for 24 hrs with 10 nM of reduced glutathione where the varying inhibitor concentrations used of the above mentioned total protein lysates were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data are mean ±SD. Data represents mean ± SEM of 5 experiments.

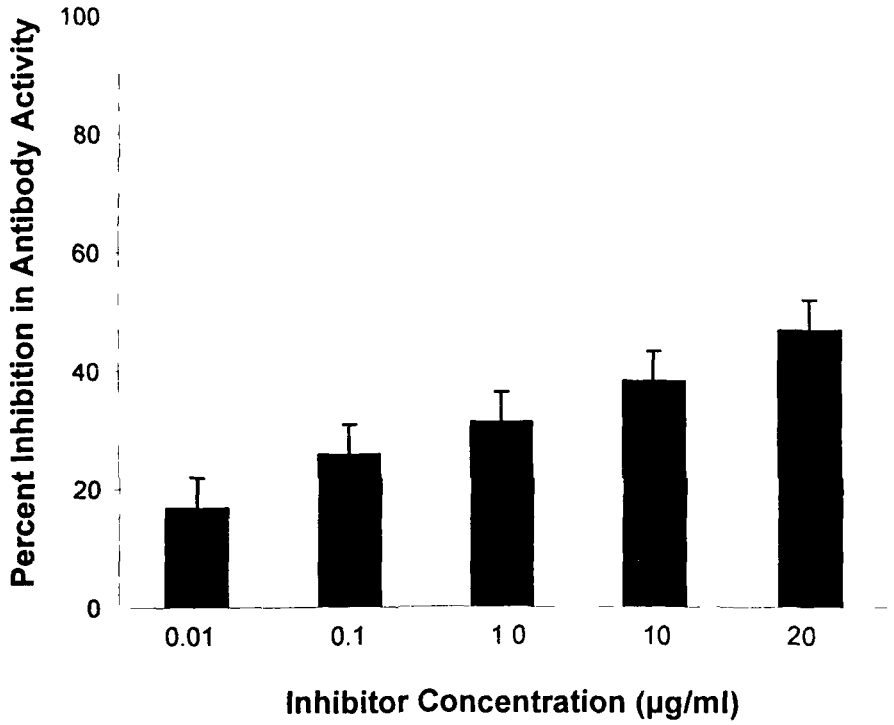


Fig. 35. Modulation of TB-SLE monocytes with reduced glutathione: Inhibition ELISA against antibodies in sera of patients with TB-SLE (n=5) on plates coated with total protein lysates obtained from TB-SLE patient monocytes that were co-cultured for 24 hrs with 10 nM of reduced glutathione where the varying inhibitor concentrations used of the above mentioned total protein lysates were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

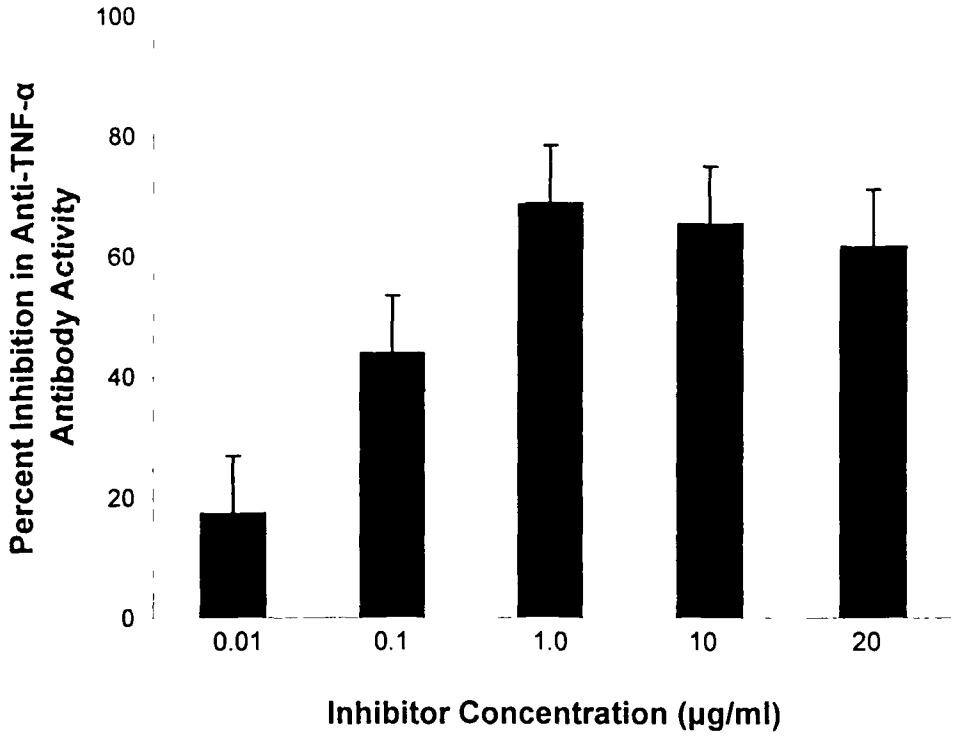


Fig. 36. Anti-TNF-α antibody Activity: Inhibition ELISA against monoclonal anti-TNF-α antibody on plates coated with total protein lysates obtained from human monocytes isolated from blood of TB patients (n=5) where the inhibitor used were varying concentrations of the above mentioned total protein lysates obtained from monocytes of TB patients. The inhibitor concentrations were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

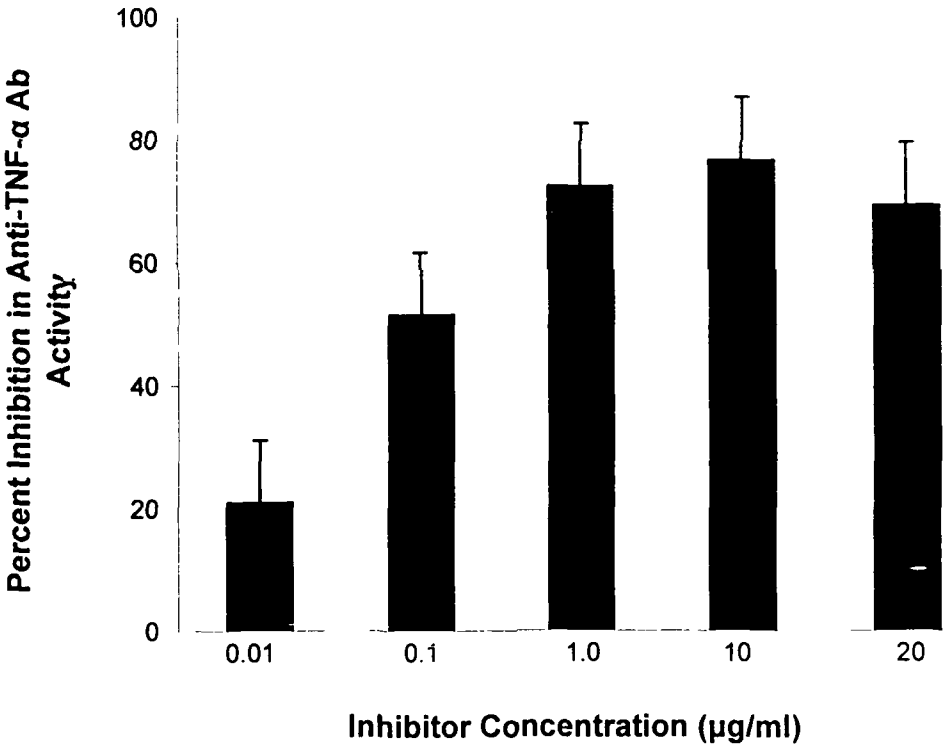


Fig. 37. Anti-TNF-α antibody activity: Inhibition ELISA against monoclonal anti-TNF-α antibody on plates coated with total protein lysates obtained from monocytes isolated from blood of SLE patients (n=5) where the inhibitor used were varying concentrations of the above mentioned total protein lysates obtained from monocytes of SLE patients. The inhibitor concentrations were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

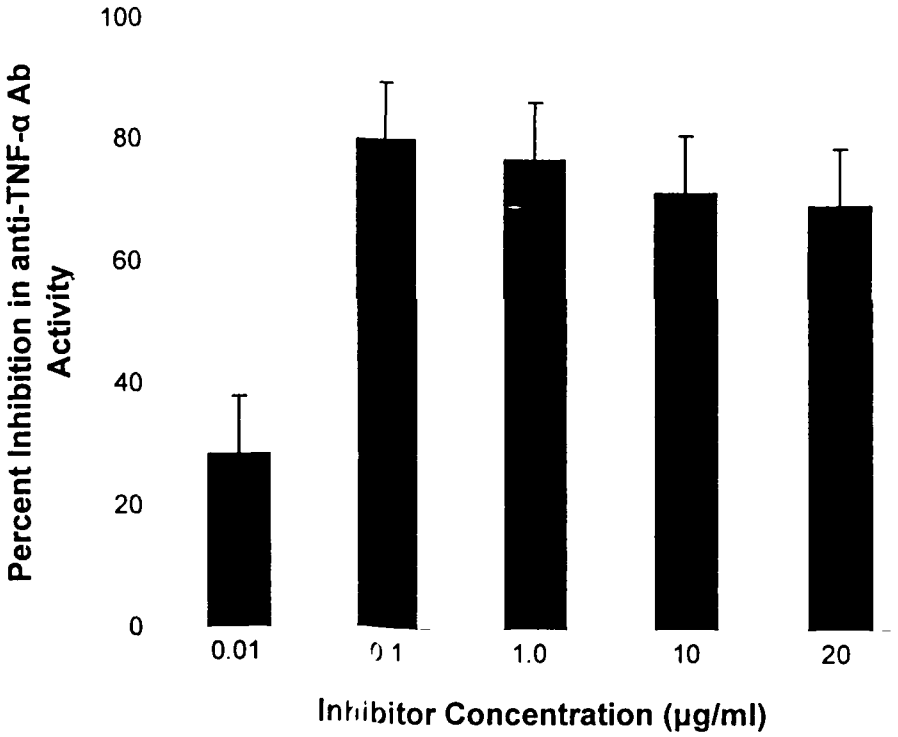


Fig. 38. Anti-TNF-α antibody activity: Inhibition ELISA against monoclonal anti-TNF-α antibody on plates coated with total protein lysates obtained from monocytes isolated from blood of TB-SLE patients (n=5) where the inhibitor used were varying concentrations of the above mentioned total protein lysates obtained from monocytes of TB-SLE patients. The inhibitor concentrations were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

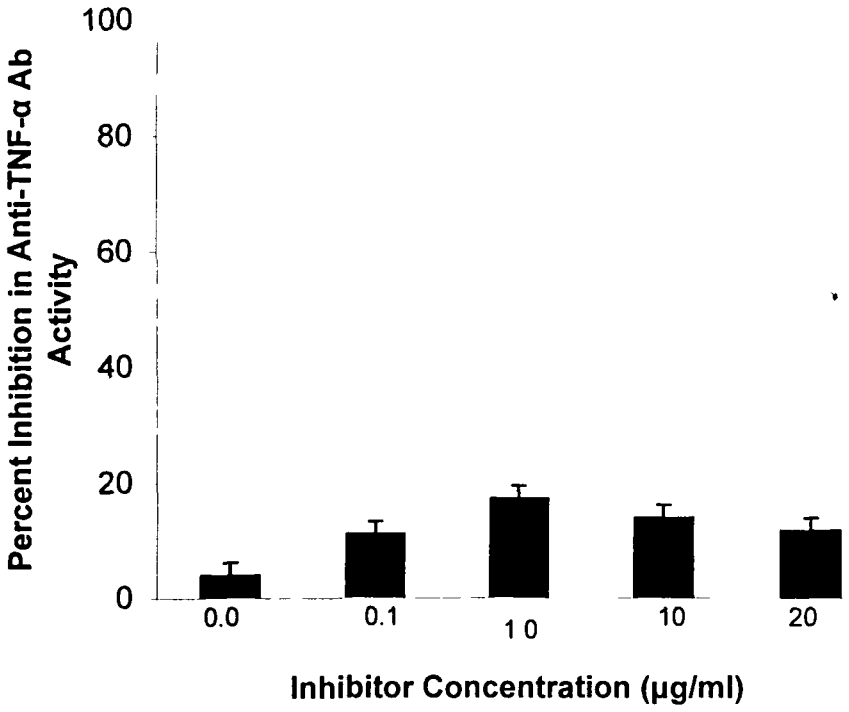


Fig. 39. Modulation of TB monocytes with reduced glutathione versus Anti-TNF-α antibody Activity: Inhibition ELISA against antibodies in sera of patients with TB (n=5) on plates coated with total protein lysates obtained from TB patient monocytes that were co-cultured for 24 hrs with 10 nM of reduced glutathione where the varying inhibitor concentrations used of the above mentioned total protein lysates were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

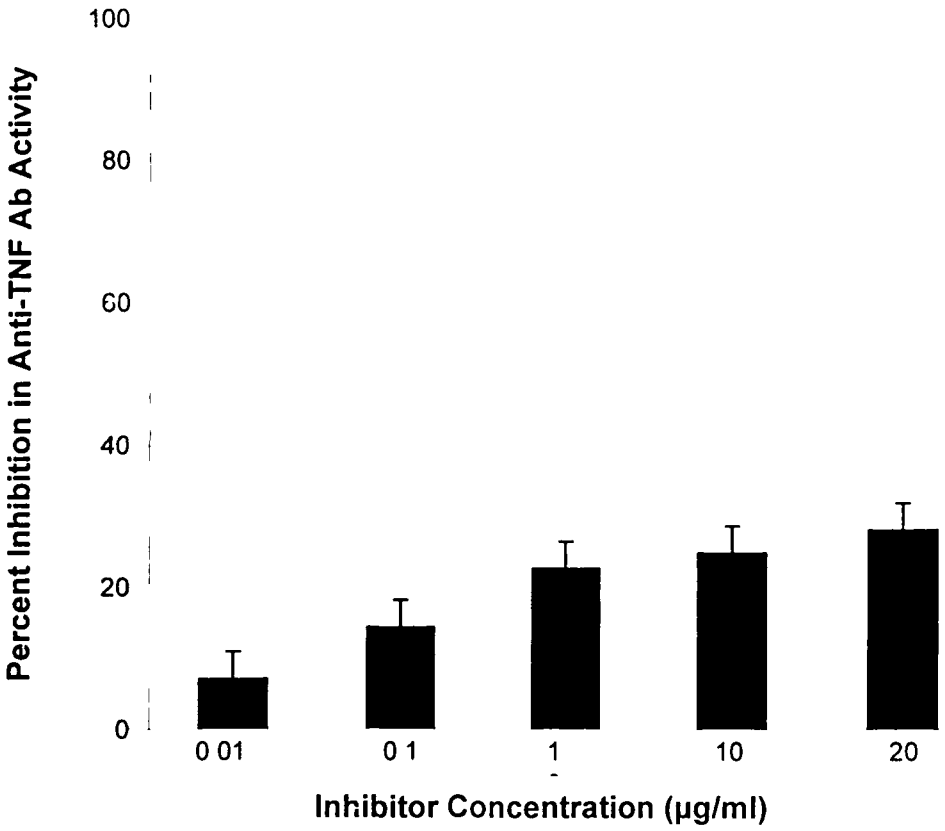


Fig. 40. Modulation of SLE monocytes with reduced glutathione versus Anti-TNF-α antibody Activity: Inhibition ELISA against antibodies in sera of patients with SLE (n=5) on plates coated with total protein lysates obtained from SLE patient monocytes that were co-cultured for 24 hrs with 10 nM of reduced glutathione where the varying inhibitor concentrations used of the above mentioned total protein lysates were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5 experiments.

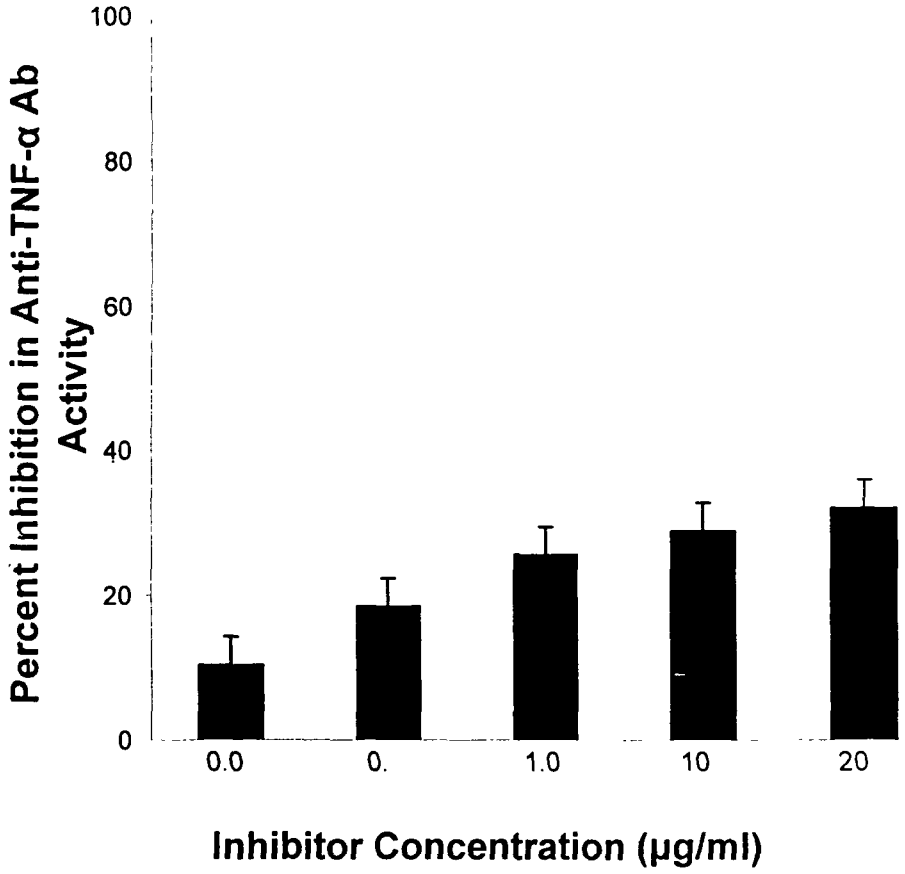


Fig. 41. Modulation of TB-SLE monocytes with reduced glutathione versus Anti-TNF-α antibody Activity: Inhibition ELISA against antibodies in sera of patients with TB-SLE (n=5) on plates coated with total Protein lysates obtained from TB-SLE patient monocytes that were co-cultured for 24 hrs with 10 nM of reduced glutathione where the varying inhibitor concentrations used of the above mentioned total protein lysates were 0.01, 0.1, 1.0, 10 and 20 µg/ml. Data represents mean ± SEM of 5experiments.

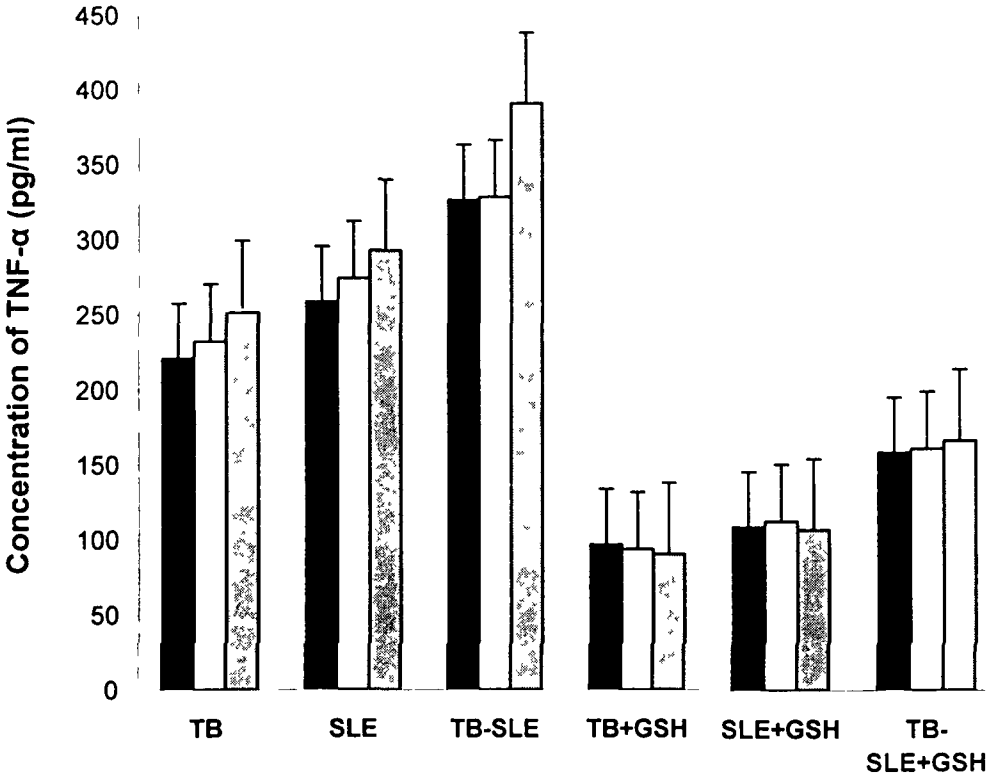


Fig. 42. Expression of secreted pro-inflammatory cytokine TNF-α in supernatants of monocytes that were cultured for 24 hrs with and without 10 nM of reduced glutathione. The different monocytes cultured were obtained from blood of patients having TB (n=5), SLE (n=5) and TB-SLE (n=5). Also in each group, three categories were undertaken in this study. They were: Category I: Patients in the age group of 20 yrs to 35 yrs (black bars), Category II: patients in the age group of 36 yrs to 50 yrs (white bars) and Category III: patients in the age group of 51 yrs to 65 yrs (shaded bars). Data represents mean ± SEM of 5 experiments.

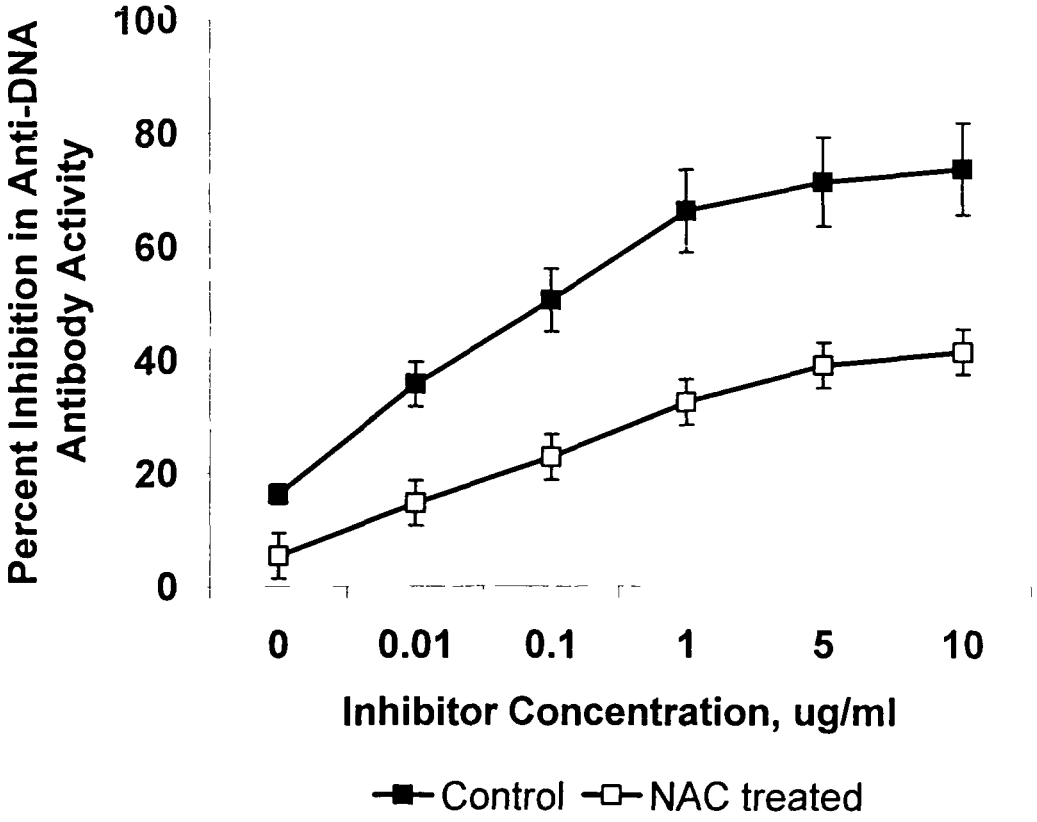


Fig. 43. Inhibition ELISA against anti-DNA antibodies in sera of patients with SLE (n=5) on plates coated with dsDNA obtained from SLE patient monocytes (n=5) that were co-cultured for 24 hrs with without (-■-) or with 10 mM of NAC (-□-), where the varying inhibitor concentrations used were 0, 0.01, 0.1, 5 and 10 μg/ml. Data represents mean ± SEM of 5 experiments. $P<0.001$ for all.

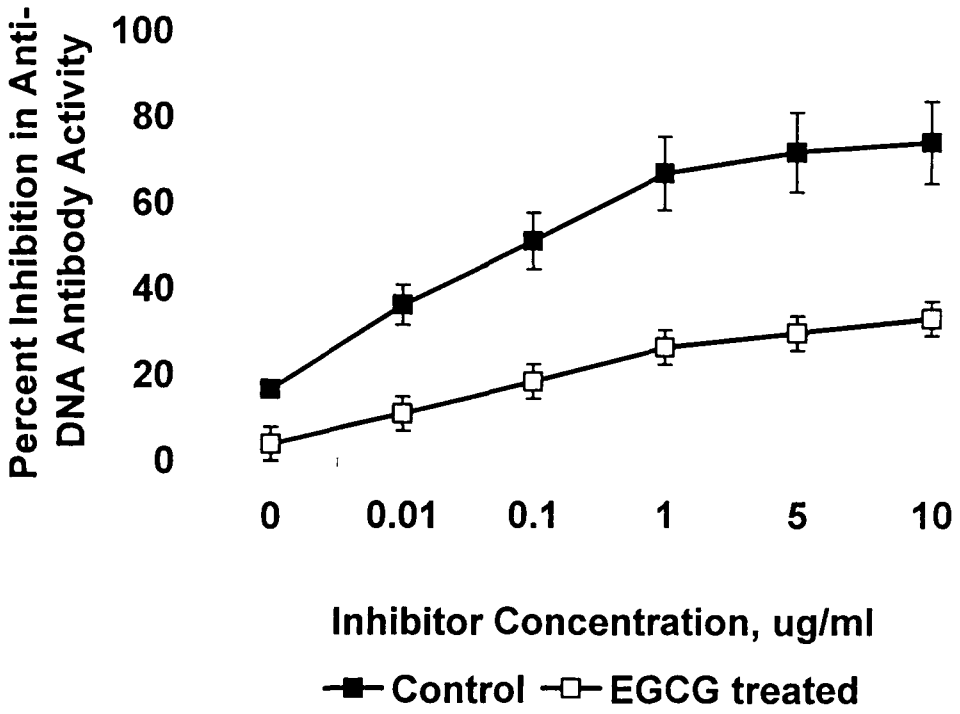


Fig. 44. Inhibition ELISA against anti-DNA antibodies in sera of patients with SLE (n=5) on plates coated with dsDNA obtained from SLE patient monocytes (n=5) that were co-cultured for 24 hrs with without (-■-) or with 5 ug/ml of EGCG, (-□-), where the varying inhibitor concentrations used were 0, 0.01, 0.1, 5 and 10 µg/ml. Data represents mean ± SEM of 5 experiments. $P<0.001$ for all.

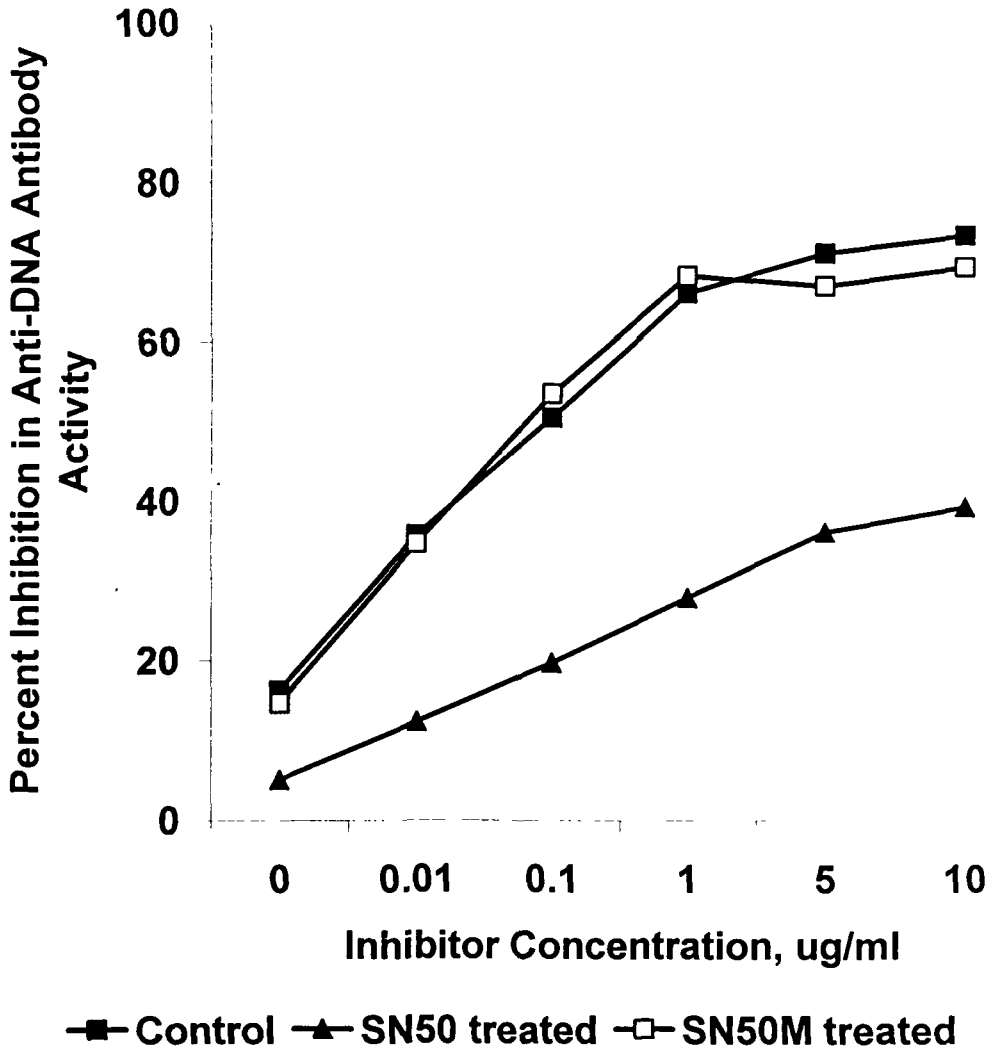


Fig. 45. Inhibition ELISA against anti-DNA antibodies in sera of patients with SLE (n=5) on plates coated with dsDNA obtained from SLE patient monocytes (n=5) that were co-cultured for 24 hrs with without (-■-) or with 100 ug/ml SN50 (-▲-) or 100 ug/ml SN50M (-□-), where the varying inhibitor concentrations used were 0, 0.01, 0.1, 5 and 10 µg/ml. Data represents mean ± SEM of 5 experiments. $P<0.001$ for all.

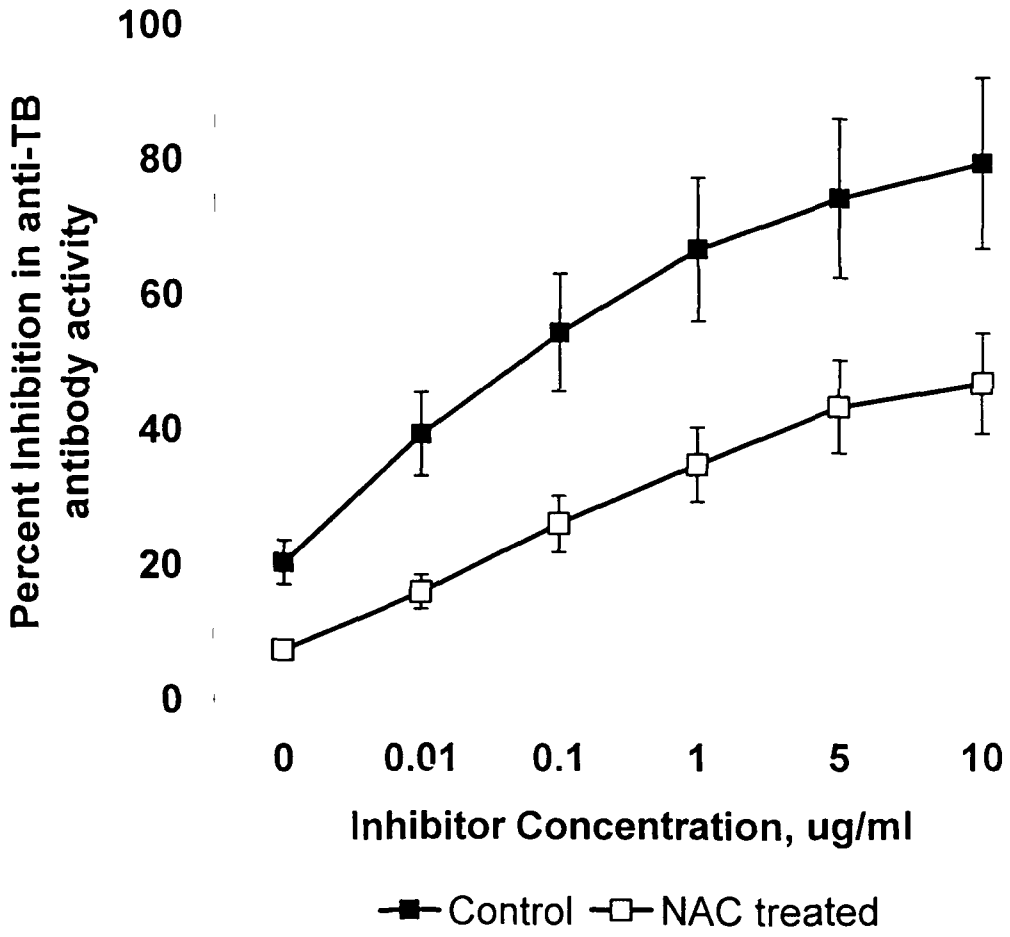


Fig. 46. Inhibition ELISA against anti-DNA antibodies in sera of patients with SLE (n=5) on plates coated with dsDNA obtained from TB patient monocytes (n=5) that were co-cultured for 24 hrs with without (-■-) or with 10 mM of NAC (-□-), where the varying inhibitor concentrations used were 0, 0.01, 0.1, 5 and 10 µg/ml. Data represents mean ± SEM of 8 experiments. $P<0.001$ for all.

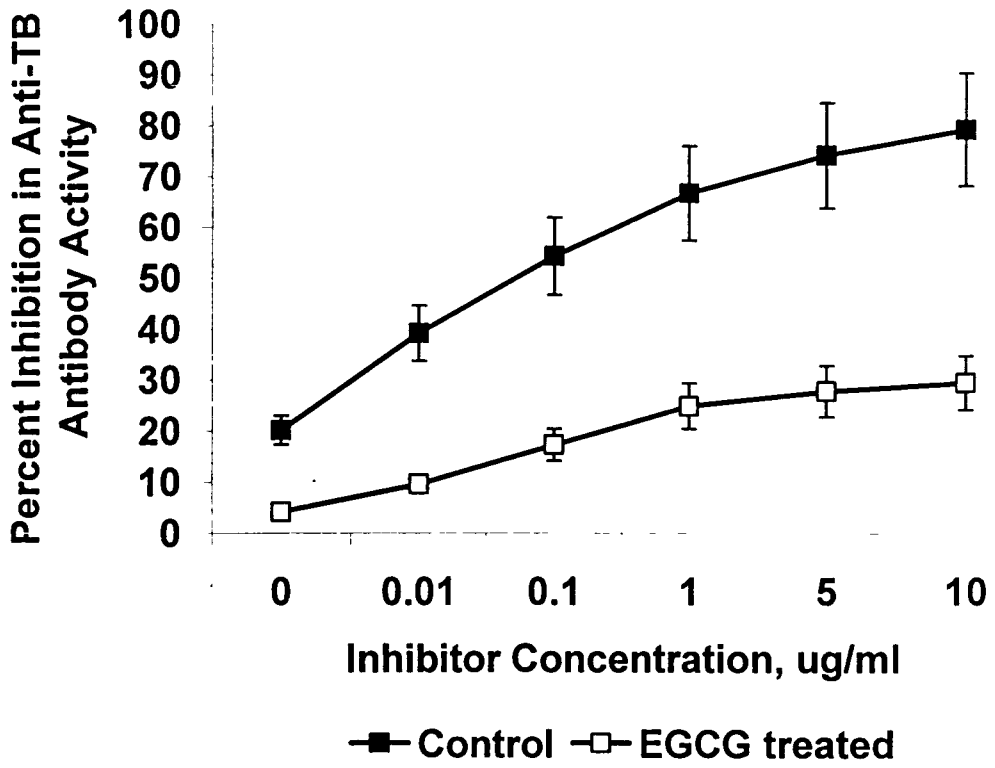


Fig. 47. Inhibition ELISA against anti-DNA antibodies in sera of patients with SLE (n=5) on plates coated with dsDNA obtained from TB patient monocytes (n=5) that were co-cultured for 24 hrs with without (-■-) or with 5 ug/ml EGCG (-□-), where the varying inhibitor concentrations used were 0, 0.01, 0.1, 5 and 10 µg/ml. Data represents mean ± SEM of 5 experiments. $P<0.001$ for all.

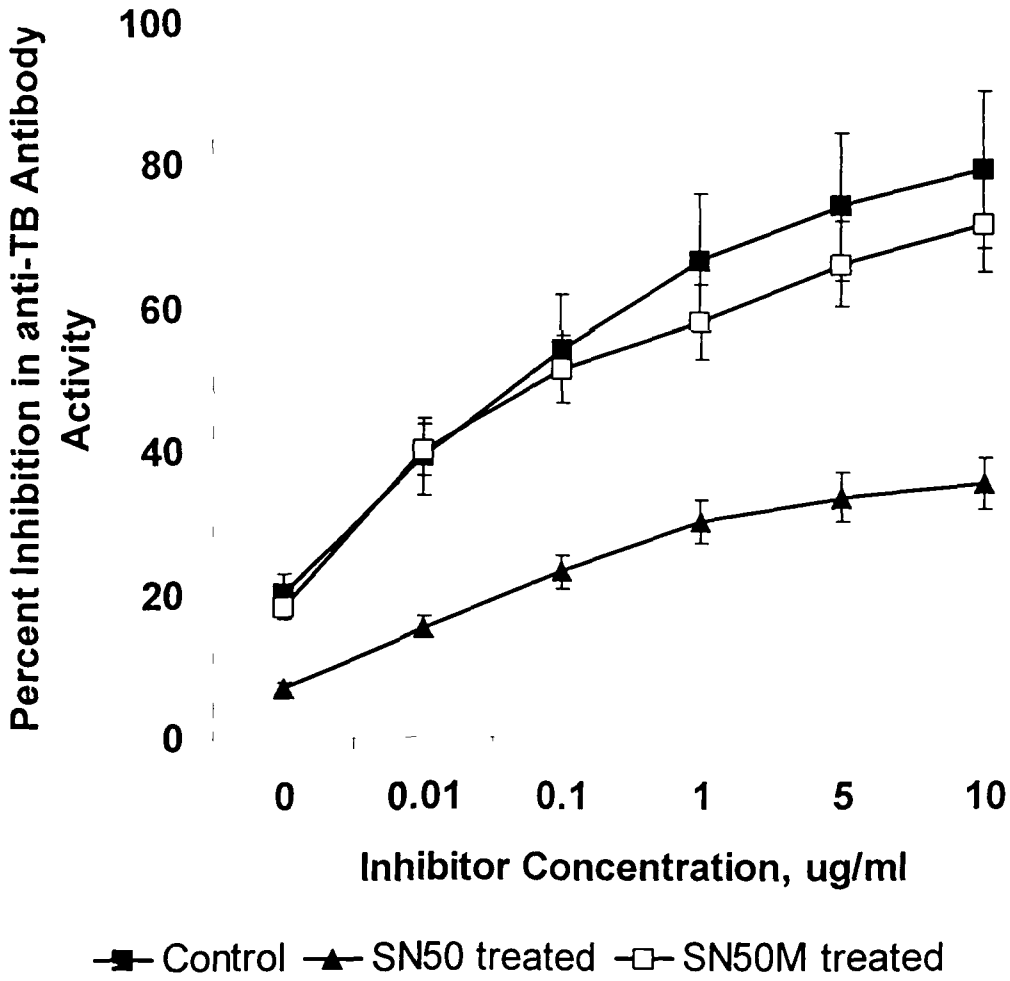


Fig. 48. Inhibition ELISA against anti-DNA antibodies in sera of patients with SLE (n=5) on plates coated with dsDNA obtained from TB patient monocytes that were co-cultured for 24 hrs with without (-■-) or with 100 ug/ml SN50M (-□-) or 100 ug/ml SN50 (-▲-), where the varying inhibitor concentrations used were 0, 0.01, 0.1, 5 and 10 µg/ml. Data represents mean ± SEM of 5 experiments. $P<0.001$ for all.

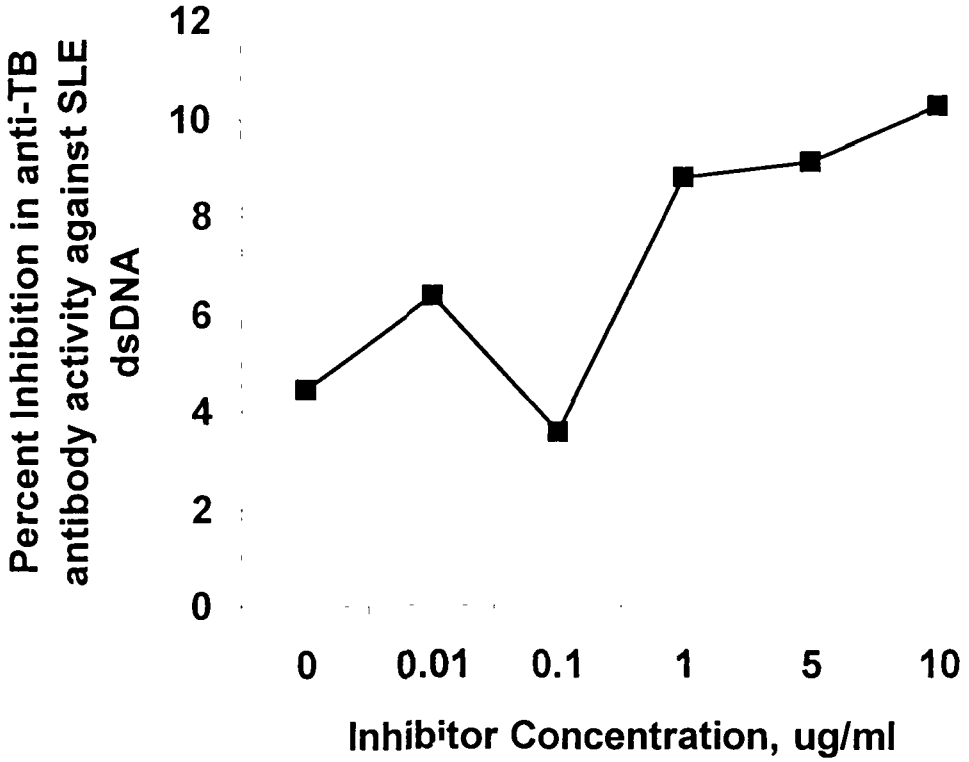


Fig. 49. Inhibition ELISA against anti-TB antibodies in sera of patients with TB (n=5) on plates coated with dsDNA obtained from untreated SLE patient monocytes (n=5) that were cultured for 24 hrs, where the varying inhibitor concentrations used were 0, 0.01, 0.1, 5 and 10 µg/ml. Data represents mean ± SEM of 5 experiments. $P<0.05$ for all.

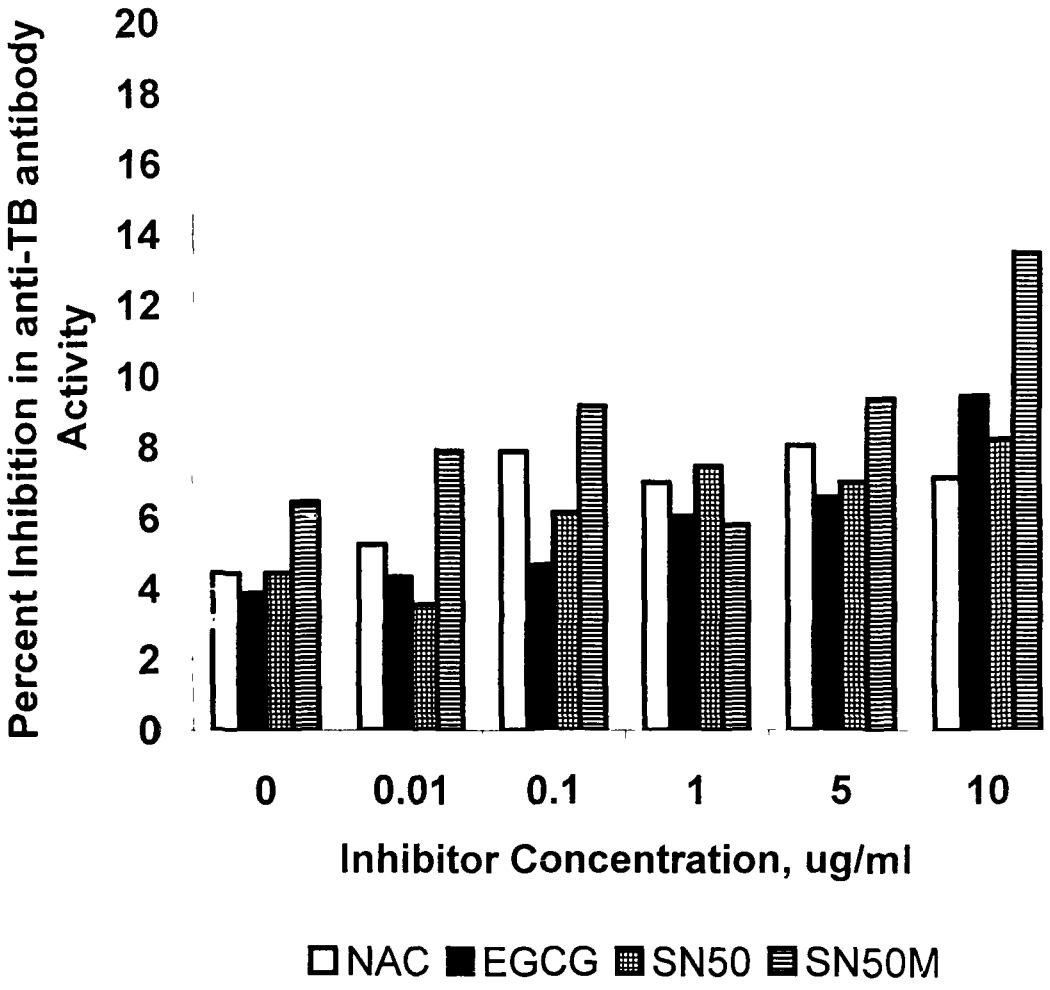


Fig. 50. Inhibition ELISA against anti-TB antibodies in sera of patients with TB (n=5) on plates coated with dsDNA obtained from SLE patient monocytes (n=5) that were co-cultured for 24 hrs with either 10 mM NAC (empty bars), 5 µg/ml EGCG (black bars), 100 µg/ml SN50 (square bars) and 100 µg/ml SN50M (ladder bars). The varying inhibitor (above respective dsDNA from treated cells) concentrations used were 0, 0.01, 0.1, 5 and 10 µg/ml. Data represents mean ± SEM of 5 experiments. $P<0.05$ for all.

DISCUSSION

DISCUSSION

The immune system has tremendous discrimination potential of self and non-self. However, this enormous recognition potential includes possible interaction with self components, some of which may appear to be essential for the regulation of the immune functions (Alam et al., 1992) whereas others can be pathogenic, particularly if expressed at high level, leading to autoimmune diseases.

Systemic Lupus Erythematosus (SLE) is a chronic, usually life-long, potentially fatal prototype autoimmune disease characterized by unpredictable exacerbations and remissions with protean clinical manifestations. In SLE there is a predilection for clinical involvement of the joints, skin, kidney, brain, serosa, lung, heart and gastrointestinal tract. SLE is a complex disorder affecting a predominately young population and shares similarities with HIV infection as regards the propensity for multiple organ involvement, potentially life-threatening episodes, and need for sophisticated monitoring. The clinical features of SLE are protean and may mimic infectious mononucleosis, lymphoma, or other systemic disease. The origin of autoantibody production in SLE is unclear but a role has been suggested for an antigen driven process, spontaneous B-cell hyper-responsiveness, or impaired immune regulation. Thus, the etiology of SLE remains unknown and warrants in-depth investigation for the management of the disease. Evidences exist for the involvement of mycobacterial protein antigens in the pathogenesis of autoimmune diseases (Tasneem et al., 2001). Despite current treatment regimens, tuberculosis continues to confound attempts at control, fuelling an urgent need for developing novel therapeutic strategies.

Although attenuation of the biological activity of TNF-α has lately become an important therapeutic intervention in the management of a wide variety of chronic inflammatory diseases (Shanahan and St Clair, 2002), a growing body of clinical evidence indicates that neutralization of TNF-α is associated with an increased risk of opportunistic infections, including mycobacterial diseases (Dinarello,

2003), and autoimmune disorders. In view of this, modulation of TNF-α release is being proposed as the basis for novel therapeutic approaches (Warwick-Davies et al., 2001). Focus has now shifted to development of compounds from natural sources that have anti-inflammatory and antimycobacterial activity. By boosting host immunologic responsiveness, these compounds may be particularly useful in the treatment of autoimmune disorders as well as drug-resistant tuberculosis. Our study involves the incorporation of such a compound, namely EGCG – a green tea polyphenol, as the natural herbal component for SLE and SLE-TB management.

Reports indicate TNF-α exerting its effects through its receptors namely TNFR-I and TNFR-II is a central mediator of inflammation (Islam et al., 2004), immunity and autoimmunity and that; it plays a crucial role in host defense. Inhibition of TNF-α clearly predisposes to certain infections, such as granulomatous infections like TB. Inhibition of TNF-α may also play a role in autoimmunity (Malemud et al., 2003; Islam et al., 2002) although the pathophysiologic mechanisms are uncertain. Furthermore, cases of TB have been reported in patients treated with TNF-α antagonists. The risk for TB in RA/SLE patients is associated with multiple other factors including age, country of origin or current residence, exposure history to persons with TB, concomitant therapy with other immunomodulators including corticosteroids, and disease activity. TNF-α antagonists have been associated with an increase in the percentage of RA patients with positive serologies (ANA and anti-ds-DNA) and lupus-like syndromes.

Cellular signalling by TNF-α is mediated mainly through activation of NF-κB (Baeuerle and Baltimore, 1996). In turn, activation of NF-κB and other pathways sustain TNF-α activity (Ropert et al., 2001). During characterization studies in order to assess the role of NF-κB in the expression of TNF-α in SLE, TB and SLE-TB monocytes, we employed SN50, an inhibitor of NF-κB. From our results,

it was apparent that the induction of TNF-α expression was mediated through activation of NF-κB, because TNF- α was suppressed when SN50 was present in cultures. The inactive analogue of SN50 (i. e. SN50M) did not have any effect. Thus, as previously reported in macrophages (Islam et al., 2004), this study also shows that cellular activation is associated with augmentation of expression of both TNF-α in monocytes of patients with SLE, TB and SLE-TB.

Putative and effective host defense mechanisms by innate immune cells to *M. tuberculosis* within the phagolysosomes of activated macrophages include the production of reactive oxygen (ROI) and reactive nitrogen intermediates (RNI). Phagocytosis of microbes as well as cellular activation activates ROIs (Takao et al., 1995), and H_2O_2, a product of the ROI pathway, activates the expression of iNOS and production of NO (Han et al., 2001). Both ROI and RNI are downstream mediators of macrophage-activating cytokines and are thought to be microbicidal. Activation of iNOS and production of NO may be important in the final containment of *M. tuberculosis* by macrophages (MacMicking et al., 1997). However, *M. tuberculosis* has evolved resistance mechanisms against both, ROI (Hillas et al., 2000) and RNI (St John et al., 2001).

Cell viability and potential cytotoxicity of EGCG, if any, on monocytes obtained from patients with SLE, TB and SLE-TB were determined for the concentrations (0-25 μg/ml) employed in this study using trypan blue and MTT assays, where viability of ~98-99% was observed. Interestingly, no effect of EGCG was observed on human housekeeping genes like R18 rRNA, thereby demonstrating that the effect of EGCG was not mediated by cellular death, but rather by specific inhibition of expression as well as secretion of TNF-α.

To the best of our understanding, in our characterization studies, we show for the first time that EGCG exerts potent anti-inflammatory effects on host mononuclear cells obtained from patients of SLE, TB and SLE-TB, as evidenced by a strong inhibition of the pro-inflammatory cytokine TNF-α. The results indicate an

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GPx catalyzes the removal of hydrogen peroxide (Mesiter and Anderson, 1983). Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms (Rukmini et al., 2004). In the present study, reduced / suppressed GPx activity in patient monocytes that were untreated or treated with SN50/M was recorded, thereby concurring with earlier reports that substantial amounts of ROS are being generated in patient cells due to cellular activation (Islam et al., 2004). Enhancement of GPx activity in monocyte cultures from patients with SLE, TB and SLE-TB after addition of NAC, a precursor of the *in vivo* antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, GPx activity was observed to be further augmented when EGCG was co-cultured instead of NAC, indicating EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in mononuclear phagocytes. Moreover, in continuation to the above, our data further shows the lipid peroxidation-induced augmented MDA levels in culture supernatants of monocytes from patients with SLE, TB and SLE-TB were appreciably suppressed or down-regulated with EGCG.

Our results are strongly indicative for the appreciable correlation between autoimmune diseases like SLE and *Mycobacterium tuberculosis* infection. Inhibition ELISA results strongly suggests that all the TB positive sera selected in this study were having a high degree of specificity towards MTB Ag85B (30kDa). Similarly, our data show high degree of recognition of anti-DNA antibodies found in SLE sera by native dsDNA. The achievement of 50% inhibition in antibody activity in both the above diseases i. e. TB and SLE at a very low inhibitor concentrations are indicative for the presence of highly specific respective antibodies in all the sera selected for this study.

One of the interesting findings of this study was that the SLE patients were found to be more susceptible to TB development than the vice-versa case. This is evident from the data indicating that autoantibodies found in SLE sera strongly recognized both native dsDNA as well as mycobacterial Ag85B 30kDa, whereas

anti-TB antibodies found in TB sera strongly recognized only Ag85B 30kDa, whereas it exhibited low or negligible recognition with native dsDNA. However, antibodies found in sera of patients suffering with both TB along with SLE (SLE-TB) revealed high specificity for both the antigens i. e. native dsDNA and MTB 30 kDa.

An attempt was also made to evaluate the immuno-binding by generating in-vivo conditions i. e. by using cultured monocytes that were infected with bacilli (TB patients) as well as monocytes from SLE patients. The high percent maximum inhibitions as well 50% inhibitions in anti-TB and anti-DNA activities at extremely low inhibitor concentrations are suggestive for the appreciably high affinity immuno-interaction occurring between anti-TB antibodies and anti-DNA autoantibodies with antigens in monocyte protein lysates as well as respective dsDNA of TB patients and SLE patients respectively.

A striking finding in the present study is the EGCG as well as reduced glutathione-induced down-regulation in binding of anti-TB antibodies and anti-DNA antibodies with antigens present in protein lysates prepared from monocyte of TB patients and SLE patients respectively. Similar actions of reduced glutathione in monocytes obtained from blood of patients having both TB along with SLE further substantiates the antioxidant-induced suppression in immuno-binding. Furthermore, these results are correlating appreciably with the amount of TNF-α secreted in different monocyte cultures undertaken in this study. The importance of glutathione and its involvement in the above actions in monocytes of TB and SLE patients undertaken in our study could be best viewed by the following mentioned relevance of glutathione. Glutathione (g-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor, which often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants. The reducing power of GSH is a measure of its free-radical scavenging, electron-donating, and sulfhydryl-donating capacity. Reducing power is also the key to the multiple

actions of GSH at the molecular, cellular, and tissue levels, and to its effectiveness as a systemic antitoxin (Meister et al., 1994). Experimental GSH depletion can trigger suicide of the cell by a process known as apoptosis (Slater et al., 1995). Antioxidants are the body's premier resource for protection against the diverse free radical and other oxidative stressors to which it invariably becomes exposed (Cross et al., 1987). The antioxidant defense system is sophisticated and adaptive, and GSH is a central constituent of this system. Nowhere is its presence more important than in the mitochondria. The liver seems to have two pools of GSH; one has a fast turnover (half-life of 2-4 hours), while the other is avidly retained with a half-life of about 30 hours (Meister et al., 1995). The first corresponds to cytosolic GSH, the second mainly to mitochondrial GSH which is known to be more tightly held.

Being directly in the path of airborne materials, the lung tissue is particularly at risk from oxidative stressors such as cigarette smoke, atmospheric pollutants, and other inhaled environmental toxins (Kidd, 1985). GSH and GSH-associated enzymes present in the epithelial lining fluid (ELF) of the lower respiratory tract may be the first line of defense against such challenges (Deleve and Kaplowitz, 1990; Pacht et al., 1991). Sustained oxidative challenge to the lung results in depletion of GSH and other antioxidants from the lungs.

As with other cell types, the proliferation, growth, and differentiation of immune cells is dependent on GSH. Both the T and the B-lymphocytes require adequate levels of intracellular GSH to differentiate, and healthy humans with relatively low lymphocyte GSH were found to have significantly lower CD4 counts (Kinscherf et al., 1994). Intracellular GSH is also required for the T-cell proliferative response to mitogenic stimulation, for the activation of cytotoxic T "killer" cells (Droge et al., 1994), and for many specific T-cell functions, including DNA synthesis for cell replication, as well as for the metabolism of interleukin-2, which is important for the mitogenic response (Wu et al., 1994).

Experimental depletion of GSH inhibits immune cell functions, sometimes markedly (Fidelus and Tsan, 1987), and in a number of different experimental systems the intracellular GSH of lymphocytes was shown to determine the magnitude of immunological capacity (Droge et al., 1994). Thus intracellular GSH status plays a central role in the functioning of immune cells. In the auto-immune diseases of systemic lupus erythematosis (SLE) and rheumatoid arthritis (RA), and as seen in aging, T lymphocytes demonstrate depressed responsiveness to antigens and mitogens, perhaps because of insufficient IL-2 production (Fidelus and Tsan, 1987). Patients with RA have low blood sulfhydryl (-SH) status (Fidelus and Tsan, 1987), as did patients with Type II diabetes or with ulcerative colitis (Fidelus and Tsan, 1987).

Chronic viral infections may also trigger GSH depletion in circulating immune cells. Patients chronically infected with hepatitis C virus have low GSH in their circulating monocytes. Monocyte GSH levels are abnormal in early HIV-1 disease (Droge et al., 1997), then in patients with advanced disease the GSH levels normalized in monocytes but the GSH/GSSG ratio became abnormal. Significant decreases in the plasma levels of both cysteine and cystine has also been documented in subjects with HIV-1 infection (Droge et al., 1997). Since cysteine is a rate-limiting precursor for GSH synthesis, an associated decrease of GSH in the lung ELF was highly suggestive of a systemic GSH insufficiency in these subjects (Buhl et al., 1989). The most marked GSH decreases occurred in subjects who were asymptomatic but had CD-4 counts below 400. Both the abnormal cytokine expression and the progression to weight loss seen in HIV-1 disease may be linked (at least in part) to abnormalities in the uptake of GSH precursors by immune cells of HIV-1 subjects, and/or to abnormalities in their synthesis of GSH.

To have further in-sight, the present study involves utilization of dsDNA isolated from monocyte cultures of SLE, TB and SLE-TB patients that were either untreated or treated with EGCG, NAC, SN50 and SN50M, an in turn, employed

as antigens / inhibitors in immunoassays against anti-DNA SLE antibodies and anti-TB antibodies respectively. Thus, interesting important observations were made. We observed that purified anti-DNA antibodies from SLE patients exhibited high degree of recognition / specificity against dsDNA isolated from monocytes of patients with SLE, TB and SLE-TB respectively. However, this high magnitude specificity / binding of purified anti-DNA antibodies from SLE patients was reduced or suppressed appreciably towards respective dsDNA isolated from monocytes of patients with SLE, TB and SLE-TB respectively that were treated with EGCG, NAC or SN50. On the contrary, anti-TB antibodies exhibited high binding only with dsDNA isolated from monocytes of patients with TB, but failed to show any significant recognition / binding with dsDNA isolated from monocytes of patients with SLE. Furthermore, when monocyte cultures of SLE, TB and SLE-TB patients that were treated with EGCG, NAC and SN50, and in turn, respective dsDNA isolated and employed in ELISA, reduced or insignificant binding was observed against anti-dsDNA antibodies from SLE patients or anti-TB antibodies from TB patients. Thus, in view of the fact that native DNA is a poor immunogen, and that, the exact trigger of anti-DNA production in SLE still remains poorly understood, where DNA has been thought to act only as a cross-reacting antigen, the results indicate possible involvement of Mycobacterium tuberculosis protein(s) / nucleic acid antigens(s) in providing an alternate trigger / origin for autoantibody production in systemic lupus erythematosus (SLE). Moreover, the data generated in the present study is suggestive for the fact that – reactive oxygen species (ROS) generated as a consequence of stress of any kind in autoimmune SLE results in the activation of proinflammatory cytokine TNF-α, which in turn results in the production of anti-DNA auto antibodies. These SLE patients having high ROS levels become highly susceptible to MTB infection. Upon MTB infection, the ROS and TNF-α potentiates or activates the MTB 85B replication in SLE-TB patients. Such an activation of ROS or TNF-α and in turn the SLE and TB proliferation could be arrested or limited by the usage of EGCG- a green tea polyphenol and reduced glutathione as revealed by our

data. In conclusion, based on characterization studies, followed by immunological data, it can be inferred from the present study that:

- There exist high basal levels of TNF-α in sera as well as in monocyte cultures of patients with SLE, TB and SLE-TB.
- Activation of monocytes by *M. tuberculosis* infection in SLE patients induces the expression of both TNF-α at both the gene and protein levels.
- Both RNI and ROI, induced early after infection of SLE monocytes, increases expression of TNF-α.
- Activation of monocytes by *M. tuberculosis* initiates a cascade of events whereby a vicious circle may exist in which expression of host inflammation and mycobacterial products amplify one another.
- EGCG (0-25 μg/ml) exhibited no toxic effect on the viability of human monocytes.
- EGCG inhibits the expression of TNF-α protein production in a dose-dependent manner in 24 hr monocyte cultures from patients with SLE, TB and SLE-TB, and that, it is mediated mainly via NF-κB.
- EGCG ameliorates the glutathione peroxidase activity in monocytes from patients with SLE, TB and SLE-TB.
- EGCG suppresses the augmented MDA levels in monocytes from patients with SLE, TB and SLE-TB.
- All the *M. tuberculosis* and SLE sera involved in this study showed a high degree of specificity for Ag85B (30 kDa) and native dsDNA respectively.
- Mycobacterial 30 kDa protein antigen (Ag85B) as well as protein

lysates prepared from monocytes of *M. tuberculosis* patients were recognized appreciably by anti-tuberculosis antibodies present in *M. tuberculosis* sera, whereas non-mycobacterial native dsDNA showed poor recognition with the same anti-tuberculosis antibodies.

- On the contrary, both non-mycobacterial native dsDNA and protein lysates prepared from monocytes of SLE patients as well as mycobacterial 30 kDa protein antigen (Ag85B) were found to be recognized appreciably by anti-DNA autoantibodies present in SLE sera.
- Furthermore, co-culturing of monocytes obtained from *M. tuberculosis*, SLE or *M. tuberculosis*-SLE with 10 nM of reduced glutathione showed amelioration of ROS and TNF-α induced actions, which in turn, subsequently suppressed the immuno-bindings observed in monocytes of *M. tuberculosis* and SLE patients cultured without glutathione.
- Our data shows that SLE patients are more susceptible to *M.tuberculosis* development, as ROS and TNF-α in SLE patients could activate the replication of Ag85B (30 kDa) after bacilli infection.
- Finally, immunoaffinity purified anti-DNA antibodies from SLE patients recognized dsDNA isolated from monocytes of both SLE and TB patients, but on the contrary, anti-TB antibodies recognized dsDNA only from monocytes from TB patients but not SLE patients.

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